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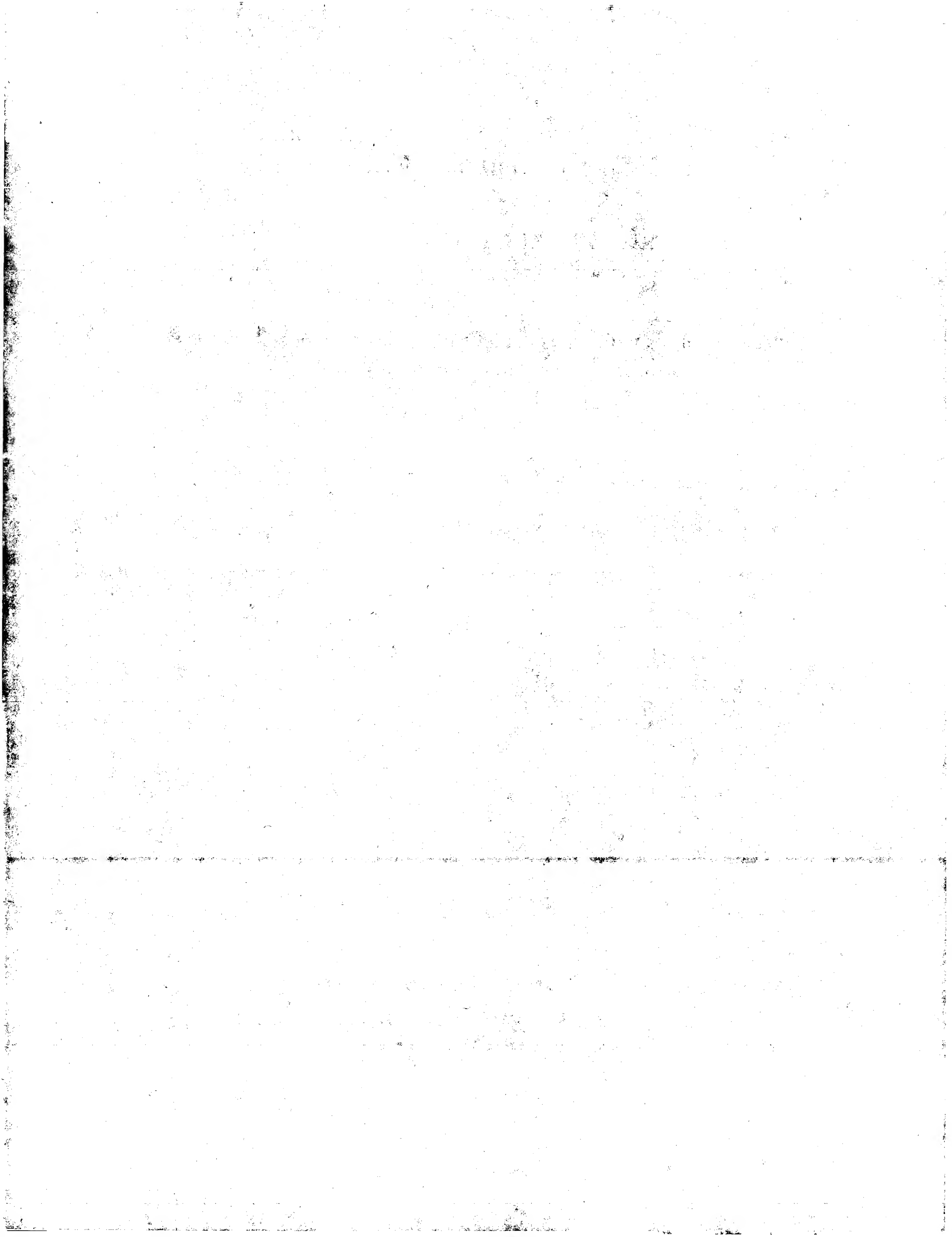
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(54) Title: SOLID PHASE DIAGNOSIS OF MEDICAL CONDITIONS (57) Abstract <p>A method of and kit for diagnosis of medical conditions by identification of specific DNA characterized in that DNA in a sample is subjected to initial amplification by the polymerase chain reaction (PCR) method using a first pair of primers specific to the target DNA and the amplified DNA so produced is further amplified by the PCR method using a second primer pair, one or both of which are different from either of said first primer pair and are specific to a sequence or sequences of the target DNA between the hybridisation sites of said first primer pair, one of the primers of said second primer pair being immobilised on a solid support or being provided with means for subsequent attachment to a solid support and the other of said second pair of primers carrying a label or being provided with means for subsequent attachment to a label, said amplification being followed by separation of said solid support carrying said amplified DNA and detection of label attached thereto, one or both of said means for subsequent attachment comprising a distal DNA sequence carried by the said primer which does not hybridise with the target DNA but has a selective affinity for a binding partner attached to either a solid support or a label.</p>		

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Solid phase diagnosis of medical conditions

5 This invention relates to a solid phase diagnosis of medical conditions by the identification of specific DNA.

10 Target DNA molecules are often present in cell lysates or other source materials in extremely small quantities and in order to amplify such DNA selectively, the polymer chain reaction (PCR) method has been developed. In this technique a pair of polymerisation primers specific to known sequences of the target DNA are selected, one hybridising at or near the 5' end of
15 the coding strand and the other at or near the 5' end of the non-coding strand such that in the presence of a polymerase, each primer produces a DNA sequence extending the full length of the target DNA template. If the DNA so produced is then subjected to strand
20 separation, typically by melting at a temperature of about 90°C, the newly formed single stranded DNA sequences will hybridise to excess primer present in the mixture, usually after reducing the temperature to the range suitable for annealing, whereupon in the presence
25 of the polymerase, further DNA strands are synthesised, this time extending only between the termini of the two primers. The polymerase is preferably capable of surviving the high temperature used in the strand separation step, a suitable thermophilic polymerase, namely Taq, having recently become available. If an
30 excess of the two primers and of nucleotides needed for DNA synthesis is maintained in the medium, it is possible to operate a repeated cyclic process in which the separate strands are synthesised, separated,
35 annealed to primer and new strands synthesised, merely by raising and lowering the temperature between the optimal temperatures

However, in such a system, it is generally the case that the primer(s) permitting immobilization to a solid support or labelling comprise a DNA sequence specific to the target DNA coupled to a non DNA grouping which has to be attached separately by chemical means.

The present invention provides, inter alia, a method whereby primer used in the final PCR amplification stage can comprise a non-DNA moiety joined to a standard or general DNA sequence which can be used in the amplification of any target DNA. This is achieved by constructing at least one of the second stage PCR primers with a 'handle' comprising an DNA sequence not hybridising to the target DNA, this handle being specific to a general DNA sequence attached to an inert support or a label so that the latter can be used in a variety of assays and does not need to be synthesised specially for a particular target DNA sequence. Although the primer(s) having a handle require synthesis to correspond to any particular target DNA, this can be completed on a standard DNA synthesis system without the need to use additional chemical methods for attaching the inert support label. It is particularly useful to be able to use radioisotopes pre-attached to a standard binding ligand or probe and thus to avoid the need for local chemical manipulation of radioactive materials.

This technique may conveniently be termed detection of immobilised amplified nucleic acids (DIANA).

According to the present invention there is provided a method of diagnosis of medical conditions by identification of specific DNA characterized in that DNA in a sample is subjected to initial amplification by the polymerase chain reaction (PCR) method using a first pair of primers specific to the target DNA and the amplified DNA so produced is further amplified by the PCR method using a second primer pair, one or both of which are different from either of said first primer

pair and are specific to a sequence or sequences of the target DNA between the hybridisation sites of said first primer pair, one of the primers of said second primer pair being immobilised on a solid support or being
5 provided with means for subsequent attachment to a solid support and the other of said second pair of primers carrying a label or being provided with means for subsequent attachment to a label, said amplification being followed by separation of said solid support
10 carrying said amplified DNA and detection of label attached thereto, one or both of said means for subsequent attachment comprising a distal DNA sequence carried by the said primer which does not hybridise with the target DNA but has a selective affinity for a
15 binding partner attached to either a solid support or a label.

Any suitable polymerase may be used, although it is preferred to use a thermophilic enzyme such as Taq polymerase to permit the above repeated temperature
20 cycle without having to add further polymerase, e.g. Klenow fragment, in each cycle.

It should be noted that the target DNA may be cDNA synthesised from mRNA in the sample and the method of the invention is thus applicable to diagnosis on the
25 basis of characteristic mRNA. Such preliminary synthesis can be carried out by a preliminary treatment with a reverse transcriptase, conveniently in the same system of buffers and bases to be used in the subsequent PCR steps. Since the PCR procedure requires heating to
30 effect strand separation, the reverse transcriptase will be inactivated in the first PCR cycle. When mRNA is the target nucleic acid, it may be advantageous to submit the initial sample, e.g. a serum sample, to treatment with an immobilised polydT oligonucleotide in order to
35 retrieve all mRNA via the terminal polyA sequences thereof. The oligonucleotide can then serve as a primer for cDNA synthesis, as described in International Patent

YMD Lo et al (Nucleic Acids Research, 16, 8719, 1988) have described carrying out PCR using biotinylated dUTP to provide amplified target DNA labelled with biotin. There is no suggestion of using the biotinyl groups for immobilisation and in this method all the U bases carry biotin groups rather than there being only terminal biotin groups.

European Patent Application 192168 of Molecular Diagnostics describes the use of dual probes for hybridisation to target DNA to provide added selectivity, one probe carrying means for immobilisation and the other carrying detection means. However, there is no suggestion of using such probes as primers in PCR amplification.

15

However, in such a system, it is generally the case that the primer(s) permitting immobilization to a solid support or labelling comprise a DNA sequence specific to the target DNA coupled to a non DNA grouping which has to be attached separately by chemical means.

The present invention provides, inter alia, a method whereby primer used in the final PCR amplification stage can comprise a non-DNA moiety joined to a standard or general DNA sequence which can be used in the amplification of any target DNA. This is achieved by constructing at least one of the second stage PCR primers with a 'handle' comprising an DNA sequence not hybridising to the target DNA, this handle being specific to a general DNA sequence attached to an inert support or a label so that the latter can be used in a variety of assays and does not need to be synthesised specially for a particular target DNA sequence. Although the primer(s) having a handle require synthesis to correspond to any particular target DNA, this can be completed on a standard DNA synthesis system without the need to use additional chemical methods for attaching the inert support label. It is particularly useful to be able to use radioisotopes pre-attached to a standard binding ligand or probe and thus to avoid the need for local chemical manipulation of radioactive materials.

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According to the present invention there is provided a method of diagnosis of medical conditions by identification of specific DNA characterized in that DNA in a sample is subjected to initial amplification by the polymerase chain reaction (PCR) method using a first pair of primers specific to the target DNA and the amplified DNA so produced is further amplified by the PCR method using a second primer pair, one or both of which are different from either of said first primer

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5 provided with means for subsequent attachment to a solid support and the other of said second pair of primers carrying a label or being provided with means for subsequent attachment to a label, said amplification being followed by separation of said solid support
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20 cycle without having to add further polymerase, e.g. Klenow fragment, in each cycle.

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30 effect strand separation, the reverse transcriptase will be inactivated in the first PCR cycle. When mRNA is the target nucleic acid, it may be advantageous to submit the initial sample, e.g. a serum sample, to treatment with an immobilised polydT oligonucleotide in order to
35 retrieve all mRNA via the terminal polyA sequences thereof. The oligonucleotide can then serve as a primer for cDNA synthesis, as described in International Patent

Application PCT/89EP/00304.

According to one embodiment of the invention the PCR procedure will be continued, after initial two-stage amplification as described above, by replacing or
5 supplementing at least one primer having such a distal DNA sequence or 'handle' by a third stage primer which hybridises to the handle so that on further amplification, the third stage primer will be incorporated into the amplified target DNA. Such a
10 third stage primer may advantageously be attached to a solid support or may carry means for attachment to a solid support or may carry a label or means for attachment of a label. As indicated above this third stage primer may advantageously be a standard DNA
15 sequence which will remain the same in all assays irrespective of the target DNA. Both second stage primers may carry 'handles' and in the said third stage of PCR amplification one such primer may be replaced by a primer hybridising to its handle section and being
20 attached to a solid support (or means for attachment thereto) while the other is replaced by a different primer hybridising to its handle and being attached to a label (or means for attachment to a label).

The label may be a radioactive atom such as ^{32}P ,
25 ^{14}C or ^3H which may be incorporated into that one of said second pair of primers. Of course, it would be possible to incorporate a radioactive nucleoside into the last polymerisation step of the second PCR and thereby add a label to the second primer. The label may
30 instead be a conventional enzyme or fluorescent substance attached directly to the primer or attached indirectly by, for example, an antibody which binds to the primer.

The means for attachment of the solid support or
35 label may be affinity binding group such as biotin/avidin or streptavidin or an amino group or a terminal nucleoside which may interact with a carboxyl

labelling of the other end enables the signal from the amplified DNA to be read after each cycle of PCR, or at intervals, thus providing data as to the extent of amplification at each stage. While the signal may be too low to be identified after the first one or two cycles, a plot of signal against PCR cycles can be extrapolated back to origin to give the initial amount of target DNA. Such a procedure requires removal of the immobilised DNA from the medium, while the signal is read, followed by subsequent reintroduction. The immobilising primer is preferably attached to the support initially so that the whole PCR procedure is effected on the support. Magnetic beads provide a particular convenient form of support for this purpose. The label is advantageously a radionuclide to permit rapid reading of the amount of immobilised label. As indicated above, the use of a universal handle for attachment of the label enables a universal radioactive label to be used.

The solid support may, for example, take the form of microtitre wells or dipsticks (e.g. plastic strips) which may be made of polystyrene activated to bind to DNA (K. Almer, Doctoral Thesis, Royal Institute of Technology, Stockholm, Sweden, 1988). Particles, fibres and capillaries made, for example, of agarose, cellulose, alginate, Teflon or polystyrene are especially convenient.

The PCR procedure according to the invention will normally be carried out in a conventional PCR buffer system. After the first phase of amplification using the outer pair of primers, it is advantageous, in addition to the new primer(s) for the second PCR phase, to add further PCR buffer to dilute the amplified product and any remaining primer molecules. In general, the dilution factor is advantageously large, for example in the range 20:1 to 200, e.g. about 100:1; however dilutions, e.g. as low as 1:1 are effective but may

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provide less selectivity.

The PCR buffer will generally be in the pH range 6.8 to 7.2, Tris/HCL is one suitable buffer. Strand separation is preferably effected at a temperature in the range 90-95°C, annealing of primers in the range 45 to 55°C and DNA synthesis in the range 65 to 75°C.

Particularly advantageously, the solid support comprises magnetic particles. Preferred magnetic particles are superparamagnetic beads produced by Dynal AS (Oslo, Norway). One of the second primer pairs may be immobilised on the magnetic particles; the particles may be already attached to the primer during the second PCR amplification stage or may be added subsequently to react with the biotinylated amplified target DNA.

Several advantages of the use of magnetic particles stand out clearly. The magnetic particles can be added to a mixture containing the target nucleic acid, e.g. a cell extract, stirred and then magnetically drawn to one side of the receptacle. The liquid can then be removed together with unwanted components and the magnetic particles, having the RNA bound thereto, can then be redispersed in a washing solution. The washing step can be repeated several times in quick succession. The whole process of obtaining the target nucleic acid can be performed in under 15 minutes.

A further advantage is the ease with which hybridisation or any process effected using the magnetic particles can be continuously monitored by magnetically aggregating the particles at intervals and assaying a label associated either with the material on the particles or with material in the supernatant.

The use of magnetic aggregation to separate the particles is far less vigorous than traditional separation techniques such as centrifugation which generate shear forces which can degrade nucleic acids or proteins.

The preferred particles are monodisperse and

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for example, if two stages of PCR provide double stranded DNA comprising the target DNA terminated with means for strongly bonding to an insoluble support at the 5'-end and a DNA handle for binding to a label via a protein such as LacI at the 3'-end, the amplified target DNA can readily be detected by means of the label, for example by an enzymatic colour reaction, and can then be subjected to strand separation to leave single stranded DNA immobilised on the inset support. The DNA thus immobilised can then be subjected to further operations. Thus for example, synthesis of a labelled second strand can be effected using a labelled primer at the 3'-end, whereupon strand separation liberates labelled ss-DNA into solution. Similarly, in vitro mutagenesis can be effected using an appropriate mutagenesis primer.

According to a particularly preferred embodiment of the invention, the immobilised single stranded DNA may be subjected to sequencing for example as described in International Patent Application W089/09282 (the contents of which are hereby incorporated herein by reference) e.g. using the method of Sanger et al (Prox. Natl. Acad.Sci. USA 1977, 74, 5462-67). Furthermore, it is possible to omit the initial detection step and carry out sequencing on the immobilised single stranded DNA finally produced by the PCR amplification stages. In both cases, the provision of a universal handle at the 3' end of the ss-DNA enables a universal sequencing primer to be used while use of the nested primer technique greatly enhances the specificity of the method and reduces background 'noise' caused by non-specific binding.

While PCR amplification enables very small amounts of target DNA to be identified and abnormalities thus diagnosed, it is difficult to obtain quantitative information concerning the amount of target DNA present in a sample, due to uncertainty as to the true extent of amplification. Immobilisation of one end of the DNA and

labelling of the other end enables the signal from the amplified DNA to be read after each cycle of PCR, or at intervals, thus providing data as to the extent of amplification at each stage. While the signal may be too low to be identified after the first one or two cycles, a plot of signal against PCR cycles can be extrapolated back to origin to give the initial amount of target DNA. Such a procedure requires removal of the immobilised DNA from the medium, while the signal is read, followed by subsequent reintroduction. The immobilising primer is preferably attached to the support initially so that the whole PCR procedure is effected on the support. Magnetic beads provide a particular convenient form of support for this purpose. The label is advantageously a radionuclide to permit rapid reading of the amount of immobilised label. As indicated above, the use of a universal handle for attachment of the label enables a universal radioactive label to be used.

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A further advantage is the ease with which hybridisation or any process effected using the magnetic particles can be continuously monitored by magnetically aggregating the particles at intervals and assaying a label associated either with the material on the particles or with material in the supernatant.

The use of magnetic aggregation to separate the particles is far less vigorous than traditional separation techniques such as centrifugation which generate shear forces which can degrade nucleic acids or proteins.

The preferred particles are monodisperse and

superparamagnetic and both these properties greatly assist the kinetics of reactions in which the particles are involved. It is a surprising feature of the invention that the probes carried by the particles react in the various reactions virtually as rapidly as if free in solution. Thus, for example, the total isolation of mRNA from a cell lysate using magnetic beads can be effected in about 15 minutes in contrast with the 2 hour period using an affinity column. By using monodisperse particles, that is particles of approximately the same size, the reaction rate and other parameters are particularly uniform. By using superparamagnetic particles (that is particles containing sub-particles of ferromagnetic material which are smaller than the domain size required to maintain permanent magnetism), one can avoid magnetic aggregation or clumping of the particles during reaction, thus again ensuring uniform and rapid reaction kinetics. Thus, the particles can readily be aggregated at a uniform speed onto a surface by application of a magnetic field but can readily be re-dispersed for a subsequent treatment step, e.g. by physical agitation. This uniformity of behaviour and rapidity of reaction lends itself particularly to automation, which is an essential requirement of many of the nucleic acid manipulations required in commercial production and/or repetitive processes. It is most important that the reactions and separations can be carried out completely reliably by an appropriate machine with minimal human intervention.

The preferred magnetic particles for use in this invention are monodisperse superparamagnetic beads produced according to EP 83901406.5 (Sintef), the disclosure of which is incorporated herein by reference. In these beads, the iron is very uniformly distributed and provides a very uniform response to a magnetic field which is important in designing a reproducible procedure, particularly for automation, since all the

beads move at the same speed. Furthermore, since a reproducible amount of iron can be incorporated in each particle, this can be adjusted to a relatively low level which permits the specific gravity of the particles to be in the range specified below. In the case of some less regular products, small particles either have too little iron to counteract Brownian forces completely when a magnet is applied or the specific gravity of the material leads to some undesirable sedimentation of the larger particles. Some automated systems use magnetic fields to restrain the particles within a reaction zone while solutions are passed through; uniform magnetic and rheological properties are essential in magnetic particles for use in such a system.

The term "monodisperse" used herein is intended to encompass size dispersions having a diameter standard deviation of less than 5%.

We prefer to use beads having a specific gravity in the range 1.1 to 1.8 most particularly 1.2 to 1.5. In the monodisperse beads used in accordance with the invention, the specific gravity is, again, particularly uniform, leading to uniform and predictable kinetic characteristics.

Advantageously, the monodisperse particles are spherical beads of diameter at least 1 and preferably at least 2 microns, being preferably not more than 10 and more preferably not more than 6 microns in diameter e.g. about 3 microns. Smaller particles sediment more slowly and in some cases the sedimentation time may be long compared to the reaction time, thus avoiding the need for physical agitation. However, particles of mean diameter 0.1 to 1.5 microns including fine particles of much smaller diameter behave less reliably in response to magnetisation.

The attachment of primers to the particles may be by direct chemical bonding as well as affinity binding, by streptavidin/biotin complexes and the like.

For attachment of the probes, the magnetic particles may carry functional groups such as hydroxyl, carboxyl, aldehyde or amino groups. These may in general be provided by treating uncoated monodisperse, superparamagnetic beads, to provide a surface coating of a polymer carrying one of such functional groups, e.g. polyurethane together with a polyglycol to provide hydroxyl groups, or a cellulose derivative to provide hydroxyl groups, a polymer or copolymer of acrylic acid or methacrylic acid to provide carboxyl groups or an aminoalkylated polymer to provide amino groups. US Patent No. 4654267 describes the introduction of many such surface coatings.

Preferred coated particles for use in the present invention may be prepared by modification of the beads according to the US Patents 4336173, 4459378 and 4654267, the disclosure of which is incorporated herein by reference. Thus, for example, macroreticular porous polymer particles, prepared from styrene-divinylbenzene and with a diameter of 3.15 microns were treated with HNO_3 to introduce $-\text{NO}_2$ groups at the surface of the pores. Then the particles were dispersed in an aqueous solution of Fe^{2+} . The Fe^{2+} is oxidised by the $-\text{NO}_2$ groups which leads to precipitation of insoluble iron oxy-hydroxy compounds inside the pores. After heating the iron exists as finely divided grains of magnetic iron oxides throughout the volume of the porous particles. The NO_2 groups are reduced by the reaction with Fe^{++} to NH_2 groups.

To fill up the pores and to introduce the desired functional groups at the surfaces, different monomers are caused to polymerize in the pores and at the surface. In the case of a preferred type of particle, the surface carries $-\text{OH}$ groups connected to the polymeric backbone through $-(\text{CH}_2\text{CH}_2\text{O})_{8-10}$ linkages. Other preferred beads carry $-\text{COOH}$ groups obtained through polymerization of methacrylic acid.

Thus, for example, the NH_2 groups initially present in the beads may be reacted with a diepoxide as described in US Patent No. 4654267 followed by reaction with methacrylic acid to provide a terminal vinyl grouping. Solution copolymerization with methacrylic acid yields a polymeric coating carrying terminal carboxyl groups as in R452 beads referred to below. Similarly, amino groups can be introduced by reacting a diamine with the above product of the reaction with a diepoxide as in the R240, R442 and R469 beads, while reaction with a hydroxylamine such as aminoglycerol introduces hydroxy groups as in the M450 and L255 beads.

Dynabeads M450 (diameter 4.5 microns) which may be obtained from Dynal, Oslo, Norway have been coated with a monomeric epoxide, resulting in a mixture of epoxy and hydroxy groups. Contact with water however, converts the epoxy groups to hydroxy groups.

Dynabeads M-280 (diameter 2.8 microns) are polystyrene beads having hydroxyl groups which have been converted into tosyloxy groups by reaction with *p*-toluene sulphonyl chloride.

Using functionalised coatings of the above types, we have found the non-specific binding of DNA and/or RNA to be very low, particularly in the case of the carboxylated beads.

The primer may be attached to the magnetic particles via carboxyl groups, the DNA being firstly provided with a 5'-terminal amino group which can be made to form an amide bond with the carboxyl using a carbodiimide coupling agent. 5'- attachment of DNA can also be effected using hydroxylated magnetic particles activated with CNBr to react with 5'- amino DNA.

The 3'-attachment of primer DNA can also be effected by chemical synthesis. Here again, the very uniform nature of the monodisperse particles provides uniform reaction rates particularly suited to synthesis in an automated synthesiser such as the Gene Assembler

(Pharmacia AS). The magnetic particle needs to be provided initially with a hydroxyl or protected hydroxyl group. Dynabeads M-280 of Dynal A/S are well suited to this purpose. If necessary, however, other surface
5 functions such as carboxyl could be used to attach a linker carrying a hydroxyl group or alternatively a 3'-attached nucleotide.

5'-Attachment may be effected by coupling of 5'-amino-oligonucleotides to tosyl-activated magnetic
10 particles. The latter may be produced by tosylation of hydroxylated magnetic particles such as Dynabeads M-280 of Dynal A/S. Displacement of the tosyloxy group leaves the 5'-amino group directly attached to the magnetic beads.

15 Where the probe is merely to be used for mRNA isolation, however, the 3' end of the probe may be attached to the magnetic particles and this may be conveniently effected by forming a phosphoramidate linkage between the 3'-phosphate grouping of the DNA and
20 an amino group on the particle.

Since biotin labelled nucleotides are commercially available, the 3'- end of DNA fragments can easily be labelled using DNA polymerase and these may be conveniently bound to avidin or streptavidin attached to
25 the magnetic particles e.g. via a hydroxy group. The biotin label may be attached to the nucleotide by a spacer arm, such as one or more ϵ -aminocaproic acid moieties, to minimize steric hindrance. Thus, for example, a double stranded plasmid may be cut at a
30 restriction site and end filled with biotinylated nucleotides, thus providing biotin at the 3'-end of each strand. If the linearised plasmid is then cut at another RE site, a section of double stranded DNA is excised and may be attached to streptavidin coated
35 beads. Removal of the non-biotinylated strand leaves a biotin-attached oligonucleotide attached to the beads.

In general, the functionalisation of the beads and

subsequent attachment of probes is advantageously such that each magnetic particle carries 10^3 - 10^6 probes.

(1-100 pmols per mg). The uniform size of the magnetic particles is of advantage in ensuring uniform probe
5 density when the probes are reacted with the particles. Uniform probe density is important in ensuring that all the probes behave in substantially the same way in the various procedures in which they are used.

It is a remarkable feature of monodisperse,
10 superparamagnetic particles that enzyme activity appears to take place very close to the particle surface e.g. within 7 bases. In the case of carboxylated Dynabeads beads it is found that the micro-surface of the beads is
15 extremely irregular, presenting an unusually large surface area which may reduce steric hinderance to hybridisation and enzyme activity close to the surface. On the other hand the non-specific binding to such carboxylated beads is not increased.

The invention also comprises kits for carrying out
20 the method of the invention. These will normally include at least the following components:

(a) a solid support such as a microtitre well or an array of such wells, a dipstick or beads, more preferably magnetic beads, the support carrying (i)
25 means for attachment to amplified target DNA, (ii) means for attachment to a primer or (iii) a primer;

(b) a label carrying (i) means for attachment to amplified target DNA, (ii) means for attachment to
30 a primer or (iii) a primer;

(c) a pair of outer primers and at least one inner primer provided with a DNA handle, where these are not attached to the support or label as in (a) or
35 (b) above;

(d) a polymerase which is preferably heat stable, for

example Taq1 polymerase;

- (e) buffers for the PCR reaction; and
- 5 (f) wash buffers for washing the support carrying immobilised DNA.

Where an enzyme label is used, the kit will advantageously contain a substrate for the enzyme and
10 other components of a detection system.

Where the support is a dipstick, this may be provided with zones capable of interacting with several different target DNA molecules to enable simultaneous diagnosis of several abnormalities to be performed.
15 Thus, such zones may carry primers specific to the respective target DNA.

The invention is illustrated by the following non-limiting Examples and Figures.

Figure 1 shows the oligonucleotides RIT1 to R7 as
20 used in Example 1.

Figure 2 shows the extra oligonucleotides RIT8 to RIT11 as used in Example 2.

Figure 3 shows diagrammatically the incorporation of biotin and a label into a sequence of target DNA as
25 in Example 1(a).

Figure 4 shows the results for Example 1(a) as a plot of relative label activity against PCR cycles.

Figure 5 shows diagrammatically the incorporation of biotin and a label as in Example 1(b).

30 Figure 6 shows the results of Example 1(b) as a plot of relative label activity against PCR cycles.

Figure 7 shows diagrammatically the incorporation of biotin and a label as in Example 1(c).

35 Figure 8 shows the results of Example 1(c) as a plot of relative label activity against PCR cycles.

Figure 9 shows the results of Example 2 as a plot of relative label activity against PCR cycles.

Figure 10 shows schematically the use of a DNA-binding fusion protein in the purification and in the detection of DNA fragments.

Figure 11 shows schematically the mutagenesis of the lacI gene.

Figure 12 shows an SDS-PAGE gel of samples from the immobilization and elution of a DNA fragment containing the lac operator sequence as described in Example 3(b).

Figure 13 shows some of the oligonucleotides for S. aureus used in Example 3(c). The handle primer RIT12 contains the lac operator sequence (as does the RIT11 primer in Figure 2) and RIT6 has a biotin 5'-end.

Figure 14 shows the results of Example 3(c) in the detection of S. aureus using PCR amplified DNA and oligonucleotides specific for the staphylococcal protein A gene.

Figure 15 shows the results of Example 3(c) in the detection of Streptococcus G148 using PCR amplified DNA and oligonucleotides specific for the streptococcal protein G gene.

Figure 16 shows the oligonucleotide primers used in Example 4 for the detection of the Pf155 gene of P. falciparum with the annealing site as described by Favaloro et al. (18)

Figure 17 shows the results of Example 4(c) in the detection of P. falciparum. Number of parasites per sample as determined by microscopy is shown. The activity is determined as the colour change at 450 nm per minute.

Figure 18 shows a schematic drawing outlining the DIANA concept for colorimetric detection of immobilized amplified nucleic acids followed by direct solid phase sequencing.

A lacI-lacZ fusion protein is used for the detection and magnetic beads as solid support.

Figure 19 shows the sequence of the CrP target gene of C. trachomatis and the primers used for detection

(RIT23-26) and sequencing (RIT43). Mutations determined by genomic sequencing of the clinical samples (Fig.22) are indicated. The numbers refer to the nucleotides as described by Clark et al (20). Note that the sequence of primers RIT24 and RIT26 are complimentary to the sequence shown. RIT43 hybridises with the lac operator sequence (-TTAACACTCGCCTATTGTTAA-5') which is introduced in the second amplification.

Figure 20 shows an agarose gel demonstrating the binding of the amplified fragment to the beads and the size of the inner and outer fragment. Lanes 1 and 2 are from material from the second amplification and lanes 3 and 4 are from the outer fragment. Lanes 1 and 3 are from unbound material while lanes 2 and 4 correspond to the supernatant after the binding to the beads. The marker is λ DNA digested with Pst I. Note that the length of the fragments is the expected size (267 and 421 base pair respectively).

Figure 21 shows the results of the DIANA assay on clinical chlamydia samples. The results of the clinical assay performed by a standard cell culture technique (35) is indicated (+/-). The activity of the DIANA is defined as described in Materials and Methods. Sample 8 is a positive control of cultivated C. trachomatis and sample 9 is a negative control for the PCR reactions (no DNA template was added to this tube).

Figure 22 shows the results of DNA sequencing of one (number 1, Fig. 21) of the positive chlamydia samples. The sequence was performed as described in Figure 18 with a general RIT43 fluorescent labelled primer. The analysis was performed on an ALF automated laser fluorescent sequencer (Pharmacia, Sweden) as described by the manufacturer.

MATERIAL AND METHODS

Bacterial strains and plasmids. Escherichia coli HB101 (1) and JM103 (2) were used as bacterial hosts. The plasmid vectors used were pSI.1 (3), pEMBL9 (4),
5 pDMI.1 (5), pNSEQ1 (6), pEZZT308 (7) and pSKS104 (8). M13K07 (9) was used as helper phage during mutagenesis. Staphylococcus aureus SA113 (10), Streptococcus G148 (11) and Bacillus subtilis 168 (12) were used in the Examples. These were grown as single colonies on
10 TBAB-plates (Difco, USA) at 37°C overnight.

A strain of C. trachomatis biovar L2 was kindly supplied by H. Gnarpe (Gävle Hospital, Sweden). Clinical samples were obtained with cotton-tipped swabs from male urethral (Karolinska Hospital, Stockholm,
15 Sweden) and stored in PCR buffer (see below "PCR amplification") at +4°C. Plasmodium falciparum parasites from patient blood samples were prepared as described by Zolg et al., (13), and the microscopic analyses were performed according to the giemsa stained
20 blood smears method (14). The strains and plasmids have been deposited at the Department of Biochemistry, Royal Institute of Technology, Stockholm, Sweden.

Synthesis of oligonucleotides

Twelve oligonucleotide primers (RIT1-RIT12),
25 complimentary to the staphylococcal protein A gene or the streptococcal protein G gene (see Figure 1, 2 and 13), were synthesized by phosphoramidite chemistry on an automated DNA synthesis machine (Gene Assembler, Pharmacia, Sweden) as described by the manufacturer. Two
30 of these primers (RIT1 and RIT6) was synthesized with an amino group in the 5 -end, which was subsequently used to introduce a biotin-derivative (15) as described by the manufacturer (Pharmacia, Sweden). RIT2, RIT7, RIT11 and RIT12 were labelled with γ ³²P-ATP (duPont, USA)
35 using T4 polynucleotide kinase (Pharmacia, Sweden) as described in Molecular Cloning: a laboratory manual (16).

Four oligonucleotide primers (RIT23-26) complementary to Chlamydia trachomatis (20), four primers complementary to *P. falciparum* (21) (RIT33-36) and a general sequencing primer (RIT43) were synthesized as described above. Two primers (RIT25 and RIT43) were synthesized with an amino group in the 5'-end, which was used to introduce a biotin (Clonetech, USA) and a fluorescent group (Pharmacia, Sweden), respectively, as described by the manufacturer. RIT25 and RIT43 were purified using a FPLC pepRPC 5/5 column (Pharmacia, Sweden).

DNA constructions

Restriction enzymes, T4 DNA ligase and Klenow DNA polymerase were used according to suppliers' recommendations (Pharmacia, New England Biolabs and Boehringer Mannheim). DNA work was performed as described earlier (16).

Protein and enzymatic assays

Cell extracts were obtained by sonication (18); the fusion protein, lacI-SPA, was purified by IgG affinity chromatography using IgG Fast Flow Sepharose (Pharmacia) as recommended and eluted, with 0.3 M HAc, pH 3.3. Fractions were collected and lyophilized prior to SDS-PAGE analysis with a 3.5% stacking gel and a 13% separating gel; performed according to Laemmli (19). The gels were stained with Coomassie blue.

Recombinants containing a functional lacZ gene were assayed by plating on X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) plates as described earlier (16). β -Galactosidase was assayed by a colorimetric procedure using ONPG (o-nitrophenyl- β -D-galactoside) in accordance with the supplier's recommendation. Alkaline phosphatase was assayed at 37°C with 15.2 mM p-nitrophenyl phosphate (Sigma No. 104-0) as substrate in a buffer consisting of 0.1 M glycine and 1 mM $MgCl_2$, pH 10.4. The streptavidin-alkaline phosphatase conjugate was supplied by Boehringer Mannheim.

PCR amplification

The in vitro amplification was carried out on a Techne Programmable Dri-block PHC-1 (Techne, UK). The general PCR solution was 1 mM of each of the primers, 200 uM of each dATP, dCTP, dGTP and dTTP, in a solution containing 67mM Tris-HCl, pH8.8, 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 6.7 mM MgCl_2 , 10mM β -mercapto- ethanol and 170 ug/ml BSA under a layer of paraffin oil. A single colony was picked from the TBAB-plate with a sterile pasteur-pipette and put into a 0.5 ml microfuge tube (Kemila, Sweden) containing 10 ul of the PCR solution adjusted to pH 10 with NaOH. The tube was transferred to the temperature block and the cells were lysed with a 5 minute heating at 95°C followed by cooling to room temperature. The solution was thereafter adjusted to pH 8.8 by adding an equal amount of a solution containing 67 mM Tris-HCl, pH7, 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 6.7 mM MgCl_2 , 10 mM β -mercaptoethanol, 170 ug/ml BSA and 1 unit of TaqI DNA-polymerase (Stratagene, USA). A temperature cycle was started consisting of the following program: denaturation of template at 92°C for 1 minute; annealing of primers at 50°C for 2 minutes and extension of primers at 72°C for 1 minute. After each fifth repeated cycle, 5 ul of solution was transferred to a new tube for binding to magnetic beads.

Binding to magnetic beads

Magnetic beads containing covalently coupled streptavidin were obtained from Dynal (Oslo, Norway). After the in vitro amplification using PCR, 5 ul of solution was added to a solution containing 10 ul 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA with 158 ug of magnetic beads. The solution was incubated for 30 minutes at room temperature and non-bound DNA was removed by repeated washes with 35 ul of 5 M NaCl followed by 10 mM Tris-HCl, 1mM EDTA, pH 7.5. A neodymium-iron-boron permanent magnet (Dynal, Oslo) was used to hold the beads in the tube during this procedure. Before the

final wash, the beads were transferred to a new tube to avoid background binding of radioactive label.

Detection of radioactive label

5 The tube containing the magnetic beads were transferred to a scintillation vial and the radiation was detected in a Scintillation Analyser (Packard, Tri-car 1500).

EXAMPLE 1

(a) Solid-phase PCR method using a single primer pair

10 The experiment shown schematically in Figure 3 was carried out using oligonucleotides RIT1 and RIT2 (Figure 1). RIT1 is biotinylated at the 5'-end, while RIT2 has been labelled at the 5'-end with ^{32}P . Three sets of experiments using different bacterial cells were carried out using these primers specific for the staphylococcal protein A gene; first S. aureus cells containing the protein A gene and then two control cells, B.subtilis and Streptococcus, lacking this gene. The results for 15 35 cycles of PCR followed by immobilization to magnetic beads are shown in Figure 4. As shown an increase of incorporated label is obtained during the PCR reaction. However, a high background is also obtained for the control samples (B.subtilis and Streptococcus G148).

20 This example illustrates that the PCR can be used with a primer pair to incorporate label into the in vitro amplified DNA fragment. However, no or little specificity is obtained due to random extension of DNA or other chromosomal DNA.

25 (b) Solid-phase rested PCR method using two nested primer pairs

30 Amplification of the specific DNA sequence was carried out according to the invention. The concept, designated dual polymerase chain reaction (D-PCR), is shown schematically in Figure 5. A pair of primers specific for a sequence of the cell to be detected is 35 used for a first round of in vitro amplification. After a suitable number of temperature cycles (e.g. 5 to 30

cycles), the sample is diluted e.g. 1:1 and a new primer pair is added. These primers are complimentary to internal parts of the primary amplified DNA fragment. The second pair has at the 5'-end either an affinity handle (such as biotin) or a label (such as an isotope). After additional PCR cycles, the amplified material is bound to a solid support and the label detected. The rationale behind the concept is that a high amount of specific template for the second PCR reaction is obtained during the first PCR reaction. This reduces the non-specific amplification of biotin and label containing DNA fragments as seen in example 1(a).

To investigate this concept as applied for specific detection of *S.aureus*, the oligonucleotides RIT3 and RIT4 (see Figure 1) were used for the first PCR reaction. However, lower concentration of primers were used (0.2 mM of each primer) and only 25 temperature cycles were performed. After 25 cycles, the mixture was diluted 1:1 with the buffer solution containing nucleotides and additional 1 unit of Taq polymerase was added together with 1 mM of the oligonucleotides RIT1 and RIT2, having biotin and ³²P at their respective 5'-ends. After various cycle times, 5 ul of PCR mixture was taken out and allowed to bind to magnetic beads carrying streptavidin.

Detection of labelled beads for the different cycles are shown in Figure 6. A low background label for the control cells (*B.subtilis* and *Streptococcus* G148), while a high degree of label is obtained for the specific cells (*S.aureus*).

This example illustrates that a high degree of labelling can be introduced into the PCR amplified material and that it can be detected using solid-phase technology described here. Low background label is achieved by the use of nested primers.

(c) Solid -phase nested PCR using handle sequence primers

A new approach to introduce solid support and label into the in vitro amplified material using a general primer pair is shown in Figure 7. The dual-PCR approach described in 1(b) is used to amplify the sequence
5 specific part of the DNA region. The second primer pair contains at each of their 5'-ends a specific handle sequence not complimentary to the DNA to be analyzed. In this way, the amplified material during the second PCR has the two different handle sequences introduced in
10 the terminal regions (see Figure 7). A third round of PCR using a general primer pair containing biotin and label, respectively, is thereafter performed. In this way, the amplified material becomes biotinylated and labelled. After binding to a solid support, the amount
15 of bound label is detected.

To test this concept, S.aureus, B.subtilis and Streptococcus G148 cells were separately used for the in vitro amplification using the oligonucleotides shown in Figure 1. RIT3 and RIT4 were used for a first round of
20 PCR as described in (b). After 25 cycles, the mix was diluted 1:1 with the buffer solution containing nucleotides and additional 1 unit of Taq polymerase was added together with 1 mM of the oligonucleotides RIT1, RIT2, RIT6, with biotin in the 5-end and RIT2 with ³²P
25 in the 5-end. After various cycle times, 5 ul of PCR mix was taken out and allowed to bind to magnetic beads containing streptavidin as described above.

Detection of labelled beads for the different cycles are shown in Figure 8. A relatively low back-
30 ground label for the control cells (B.subtilis and Streptococcus G148) is obtained, while a high degree of label is obtained for the specific cells (S.aureus) after 15 cycles.

This example illustrates that a high degree of
35 label can be introduced into the PCR amplified material in a specific manner using general biotin and label primers. Low background level is achieved although the

primers are not homologous with the DNA detected.

EXAMPLE 2

Solid-phase D-PCR detection of Staphylococci and Streptococci using the handle approach

5 D-PCR detection of specific DNA using the handle approach was further tested using oligonucleotides specific for either the staphylococcal protein A gene (Figure 1) or the streptococcal protein G gene (Figure 2). Cells of either S.aureus SA113, E.subtilis 168 or
10 Streptococcus G148 were analyzed by both sets of oligonucleotides.

The detection of the S.aureus gene was carried out as described in Example 1 except that the second round of PCR contained only three oligonucleotides; RIT5, RIT6
15 with biotin and RIT2 end labelled with ^{32}P . The detection of the Streptococcus gene was carried using the same scheme as described above using primers RIT8 and RIT9 for the first round of PCR (25 cycles) and RIT10, RIT6 with biotin and RIT11 end labelled with ^{32}P
20 for the second round of PCR. The second round of PCR was terminated after 15 cycles and the amplified material was allowed to bind to streptavidin coated magnetic beads as described in Example 1.

The results of the assay is shown in Figure 9. A
25 relatively low background label is obtained for the control cells using both sets of oligonucleotides (S.aureus specific oligonucleotides shown as filled bars and Streptococcus specific oligonucleotides shown as striped bars). For both sets of primers, a significant
30 amount of label is introduced in the in vitro amplified material when the specific cells are analyzed. This example illustrates that the concept can be used for detection of two different bacteria using the same general biotinylated handler primer in both assays.

35 EXAMPLE 3

This Example concerns the use of a DNA-binding protein to purify DNA fragments and in the detection of

PCR amplified fragments as shown schematically in Figure 10.

(a) Construction and analysis of a DNA-binding fusion protein

5 A gene fusion comprising the staphylococcal protein A (SPA) gene and the E.coli lacI gene encoding a DNA-binding repressor molecule was constructed. To enable in vitro mutagenesis of the stop codon at the end of the lacI gene (thus creating a open reading frame)
10 the gene was first cloned into pEMBL9. The donor plasmid pSI.1 was digested with PstI and BamHI giving a fragment of 1361 bp, including the lacI gene of 1080 bp, which was isolated and inserted between the same sites in the mp 9 linker of pEMBL9.

15 After transformation, E.coli JM103, was spread on plates consisting of ampicillin, X-Gal and IPTG, and white colonies were isolated. Single strand plasmid DNA, pEMBL/lacI, was obtained by transforming plasmid DNA from one of these colonies into JM103 using the
20 packing deficient helper phage M13K07 to infect and to pack single stranded DNA with the pEMBL-vector carrying the organ of replication from phage f1. By the use of a synthetic oligonucleotide,
(5'-ATTCCCGGGATCCTCTGCCOGCTTTCCAG-3') a mismatch priming
25 on the single strand template was performed. The primer extension conditions were essentially as described by Carter (17). The extended material was used to transform E.coli JM103. The mutant plasmids, pEMBL/lacI STOP, were selected by blue colonies on agar plates
30 containing ampicillin, X-Gal and IPTG. Figure 11 shows the in vitro mutagenesis schematically; the nucleotides indicated by asterisks are those which are deleted during the mutagenesis.

35 The 3'-end of the mutated lacI gene without the stop codon was released with EcoRV and Xma from plasmid pEMBL/lacI STOP yielding a fragment of 291 bp. The isolated fragment was ligated into pDMI.1, which had

been digested with the same restriction enzymes. The pDMI.1 plasmid consists of three major elements: a lacI^q gene overproducing the lac repressor, the gene for neomycin phosphotransferase conferring resistance to kanamycin and the p15A replicon. The resulting construction, pDMI.1 STOP, thus encodes mutant lacI. A fragment encoding a part of protein A was cleaved out from the pNSEQ1 vector with BamHI and isolated. This fragment was then inserted into a unique BamHI site in the 3'-end of the lacI-gene in plasmid pDMI.1 STOP plasmid. This new plasmid, pRIT34, encodes the lacI-SPA fusion gene of 1926 bp.

E.coli strain HB101 harbouring the plasmid pRIT34 was grown over night in baffled Erlenmeyer- flasks containing Tryptic Soy Broth (30 g/l) (Difco, USA) with an addition of Yeast Extract (7 g/l), and kanamycin (50 mg/l). The fusion protein, with a molecular weight of 70.6 kDA (deduced from the amino acid sequence) was purified from sonicated cells using affinity chromatography on matrix-bound IgG. After elution and lyophilizing the affinity-purified proteins were analyzed by SDS-PAGE. The production level was approximately 20 mg/l culture. Almost 90% of the affinity purified protein was found to be full-length.

This example illustrates that a lacI-SPA fusion protein can be produced in a recombinant host and that the fusion protein can be immobilized and purified on a solid support containing covalently bound IgG.

(b) Purification of DNA fragments

Plasmid DNA of pEZT308 was digested with HgiAI and NotI, yielding fragments of different length: 85, 497, 676, 1161 and 1183 bp. The fragment of 676 bp contained the lac operator sequence. After dilution of the digested DNA to a concentration of 140 ng/ul it was divided into 3 aliquotes. IgG-Sepharose was mixed with lysates of HB101 harbouring pRIT34 for 1 hour at room temperature. The lacI-SPA fusion protein present in the

lysate became immobilized on the IgG-Sepharose via the SPA moiety. After extensive washing with a washing buffer consisting of 0.1 M Tris, 0.15 M NaCl and 0.1% BSA, pH 7.4 to eliminate excess fusion protein, 100 ul of the immobilized lac-SPA fusion protein was mixed at room temperature with 100 ul of digested DNA from plasmid pEZZT308. Analysis of the supernatant revealed that the 676 bp fragment had selectively bound to the gel (Figure 12), lane 3).

After repeated washing, followed by incubation at room temperature for 45 minutes with 1 mM IPTG, resulted in elution of the fragment (Figure 12, lane 4). As shown in Figure 12 (lane 1 and 2), the negative controls using IgG-Sepharose without lysate (lane 1) or IgG-Sepharose mixed with lysates from HB101 harbouring pDMI.I (lane 2), yield no or little binding of the specific DNA to the column. (Lane M shows marker DNA)

This illustrates that the DNA-binding part of the fusion protein is functional after immobilization to the solid support through the protein-A-IgG complex. It shows that a fragment can be immobilized to the column and eluted with an IPTG solution in manner schematically outline in Figure 10.

(c) Detection of PCR amplified DNA fragments

The use of the PCR technique has made it possible to generate multiple copies of target DNA from only one DNA template. We have designed and described above oligonucleotides which are capable of hybridizing to specific DNA of the genomes of S. aureus and Streptococcus G148 and have shown their species specificities by reference to, e.g. B. subtilis. The dual-PCR approach, i.e. using with two sets of nested primers, can be used to reduce amplification of non-specific DNA fragments as shown above. Below we describe a first amplification of 25 cycles with flanking primers, followed by amplification of 15 cycles, using nested handle primers for immobilization

and detection. The handle primers were: RIT6 with biotin in 5'- end, RIT11 and RIT12 with the lac operator sequence. The primers used are schematically shown in Figures 2 and 13. The PCR amplification was carried out
5 as described in Example 2.

The procedure used is shown schematically in Figure 10B. Immobilization of the lacI-SPA fusion protein to IgG-Sepharose was carried out as described in Example 3(b), except that the washing buffer consisted of 0.1 M
10 Tris, 0.15 M NaCl, 0.1% Tween 20, 1mM MgCl₂ and 0.1 mM ZnCl₂. 100 ul of IgG-Sepharose carrying the immobilized fusion protein was mixed with 20 ul of amplified PCR-mixture (diluted with washing buffer to 500 ul) for 60 minutes at room temperature. The amplified DNA is
15 bound to the Sepharose by the interaction of the lac operator sequence of the handle with the lacI part of the lacI-SPA fusion protein. The sepharose beads were then washed 3 times with 1.5 ml of buffer. Detection of bound amplified DNA was carried out by the addition of
20 100 ul of streptavidin- alkaline phosphatase conjugate to the mixture which was left to stand for 30 minutes. The streptavidin- alkaline phosphatase is bound to the amplified DNA via the 5' biotin, as shown in Figure 10B. Excess of conjugate was eliminated by three washings
25 with 1.5 ml of washing buffer. 500 ul of alkaline buffer was then added, the temperature adjusted to 37°C, and then 500 ul of substrate was added. The enzyme reaction was stopped with 40 ul 0.5 M NaOH and the activity measured (by change of absorbence per second at
30 405 nm) using a spectrophotometer.

Figure 15 and 16 show the results of using either oligonucleotides specific for S.aureus (Figure 14) or oligonucleotides specific for Streptococcus G148 (Figure 15). A high degree of colour change above a background
35 level is achieved for both oligonucleotides. This illustrates that the immobilized fusion protein can be used to recover DNA fragments containing the lacI

operator, that the bound DNA molecules can be detected by a simple colour reaction and that the same immobilizing fusion protein colour reaction can be used in the detection of different amplified DNA fragments.

5 EXAMPLE 4

This example demonstrates that an infectious agent of great clinical importance could also be detected, the DIANA was used to determine the presence of Plasmodium falciparum DNA in clinical blood samples.

10 The two step PCR procedure outlined in Figure 10 was carried out using the five oligonucleotides shown in Figure 16. The *P. falciparum* specific primers hybridize to the 5'-end of exon II in the Pf155/RESA gene (21). This gene codes for a parasite antigen, considered to be
15 one candidate for a future malaria vaccine (22,23). The Pf155/RESA gene has only been characterized in two parasite strains earlier (21). The 5' region of the gene was chosen because it is thought to be relatively conserved in different strains, although it might
20 contain minor variations. The first set of primers generates a 428 bp fragment and the second set a 398 bp fragment in the earlier characterized strains.

The two step PCR amplification was carried out on 14 samples obtained from patients in The Gambia, Africa
25 as described in Example 3. The resulting material was captured in the LacI containing sepharose (Example 3) and detected via the streptavidin-alkaline phosphatase conjugate. The results of the assay are presented in Figure 17. Note that samples from patients with low
30 grade parasitemias were included in the study, to investigate the sensitivity of the assay. All the samples positive by microscopy, were also positive in the DIANA, using only 1 μ l sample volumes. 10 parasite genomes could reproducibly detected, with a distinct
35 signal significantly over background.

EXAMPLE 5

This example describes a system for rapid

colorimetric detection of specific genomic DNA fragments amplified by the polymerase chain reaction (PCR) which has been designed to allow for direct solid phase sequencing of positive samples. The amplified material is immobilized to magnetic beads using the biotin streptavidin system. A lac operator sequence is incorporated in the amplified material during the second step of a nested primer procedure. This 21 base pair sequence is used for a general colorimetric detection with a fusion protein consisting of the Escherichia coli lac repressor and β -galactosidase. Positive samples can subsequently be treated with alkali to obtain a single stranded DNA template suitable for direct genomic sequencing. This method to detect immobilized amplified nucleic acids (DIANA) is well adapted for automated or semi-automated clinical assays. Here, we show that it can be used to detect and sequence Chlamydia trachomatis genomic DNA in clinical samples.

(a) The basic concept

The approach for the detection and sequencing of specific in vitro amplified material using a magnetic bead as a solid phase is shown in Figure 18. The first step is a standard PCR with oligonucleotides specific for the target DNA sequence. A large number of cycles, i.e. 25-35 cycles, are performed to obtain many template molecules that can be used in the second PCR step. for samples lacking the target DNA, a non-specific amplification of random DNA will occur as described earlier. The material obtained after the first PCR step is diluted and subsequently used for a second PCR with the inner primers, which are specific for a sequence within the DNA fragment amplified in the first step. One of the primers is biotinylated, while the other contains a lac operator "handle" consisting of 21 nucleotides. Thus, successful amplification yields specific DNA with biotin and lac operator incorporated in the fragment. The second PCR reaction is carried out

with fewer cycles, 15, and therefore very low yield of fragments containing biotin and lac operator will be obtained for samples containing non-specific DNA (Fig. 18).

5 The biotinylated material is thereafter captured on magnetic beads coupled with streptavidin using interaction between biotin and streptavidin. Thus, non-biotinylated fragments can easily be removed. A recombinant fusion protein consisting of the lac
10 repressor (lacI) and β -galactosidase (lacZ) is added to the beads and bound enzyme conjugate is detected by adding chromogenic substrate, specific for the β -galactosidase enzyme.

15 The samples identified as positive using this colorimetric procedure can directly be treated with alkali to remove the bound fusion protein and to melt the double stranded DNA immobilized to the beads. The biotin streptavidin complex is resistant to this treatment and thus a single stranded template suitable
20 for sequencing is obtained using this one-step elution procedure. A solid phase DNA sequencing protocol (24) can therefore be followed without the need for extra template preparations. The extended material is finally eluted from the beads using formamide and loaded on a
25 sequencing gel. The solid phase method provides a clean template (24) which ensures that reproducible sequence information is obtained without the need for precipitations, centrifugations or desalting procedures.

(b) Design of the synthetic primers

30 Chlamydia trachomatis is Gram negative bacteria characterized by an obligatory parasite life cycle within eucaryotic host cells. On the basis of clinical, biological and molecular characteristics, the human C. trachomatis isolates have been grouped in two biovars
35 and 15 serovars (25,26) Serovars L1, L2 and L3 are clinically important as they are associated with relatively invasive form of chlamydia disease involving

lymphoid tissue (27).

Several genes from serovars L1 and L2 of *C. trachomatis* have been isolated and characterized. This includes the major outer membrane protein (MOMP) from serovar L1 (28) and L2 (29) and the outer membrane cysteine rich protein (CrP) from serovar L1 (20). We decided to perform a DIANA assay, as described in Figure 18, with *C. trachomatis* as a model system using the sequence of the CrP gene as target DNA. The Cr protein has been suggested to be necessary for the structural integrity of the chlamydial elementary body envelope (30), which makes the protein particular important given the apparent absence of peptidoglycan of *C. trachomatis* (31). Therefore, this gene is most likely essential for invasive chlamydia and a potential candidate as a target for detection of chlamydial DNA in clinical samples.

Four oligonucleotide primers specific for the middle part of the CrP coding region of *C. trachomatis* serovar L2 were synthesized. The sequence of the four primers are shown in Figure 19 where also the location of the target DNA within the CrP gene is shown. The two outer primers hybridize 421 base pairs from each other, while the inner primers are 267 base pairs apart. Biotin was covalently coupled to one (RIT25) of the inner primers, while the 21 base pair handle corresponding to the *E. coli* lac operator sequence (see Material and Method for details) was added to the 5'-end of the other inner primer (RIT26).

Immobilization of the PCR amplified material on magnetic beads.

To test the efficiency of the primers designed to amplify the chlamydia CrP coding region, a culture of *C. trachomatis* serovar L2 was used for a standard agarose assay. Cells were directly lysed in the PCR buffer and

the CrP gene was amplified for 25 cycles using the outer primers (RIT23 and RIT24, Fig. 19). After a 100-fold dilution of the obtained material, a second PCR step was performed for 15 cycles using the inner primers (RIT25 and RIT26, Fig. 19). The PCR buffer used contained 20mM TAPS (pH=9.3), 8 mM MgCl₂ and 0.2% Triton X. The clinical tops were put into PCR tubes filled with PCR buffer (10 µl) and rubbed against the wall of the tube. The tube was subsequently heated to 99°C for 5 minutes in order to lyse the cells and then cooled on ice. The lysed mixture 90 µl of a PCR buffer with 0.2 mM dNTP, 0.2 mM of each primer and 1.0 units of Taq polymerase (Boehringer Mannheim, Sweden) was added. One temperature cycle on the PCR was: denaturation of template 95°C, 0.5 minute; annealing of primers 58°C, 1 minute; extension of primers 72°C, 1 minute.

The material after the first and the second PCR step, respectively, was mixed with magnetic beads containing streptavidin and the supernatant was analyzed by agarose gel electrophoresis.

Magnetic beads with covalently coupled streptavidin, Dynabeads M280-Streptavidin (31) was obtained from Dynal (Norway). A neodymium-iron-boron permanent magnet (Dynal, Norway) was used to sediment the beads during washing procedure. 300 µg of the beads was mixed with 85 µl of the PCR mixture and incubated for 20 minutes at room temperature. The beads was then extensively washed using the TST buffer, described above.

The results (Fig. 20) shows that the first PCR yields a specific band (lane 3) of the expected size (421 base pairs), which does not bind to the magnetic beads (lane 4). In contrast, the shorter sized (267 base pairs) material obtained after the second PCR step (lane 1) is efficiently captured by the magnetic beads (lane 2). More than 95% immobilization yield is achieved, which demonstrates the efficiency of the

biotin-streptavidin system for capturing DNA on a solid support.

(c) Purification of a lacI-lacZ fusion protein

Escherichia coli XAc/ Δ 14(33), containing the lacI
5 and lacZ gene fusion encodes a fusion protein with both
LacI repressor and β -galactosidase activity. The E.coli
strain was grown overnight in baffled Erlenmeyerflasks
containing Tryptic Soy Broth (30 g/l), (Difco, USA),
with addition of Yeast Extract (7 g/l) at 37°C
10 overnight. Cell extracts were obtained by sonication
(34) in a TST buffer (0.1 M Tris-HCl, pH=7.5, 0.15 M
NaCl, 0.1% Tween) with addition of 0.1% BSA, 0.1 mM
ZnCl₂, 1.0 mM MgCl₂ and 10 mM β -mercaptoethanol. The
fusion protein was purified by (NH₄)₂SO₄ precipitation,
15 using the fraction between 25 and 45% (NH₄)₂SO₄
saturation, followed by affinity binding to Heparin-
Sephadex column (Pharmacia, Sweden). The fusion-
protein was eluted in a 0.25 M KCl and 15% glycerol
buffer.

20 (d) Colorimetric DIANA of chlamydia in clinical samples

Several clinical samples of human urogenital origin
was used to investigate the DIANA concept as outlined in
Figure 18. A standard cell culture assay (35) was
carried out in parallel on the same samples. The C.
25 trachomatis serovar L2 cell culture (Fig. 21) was used
as a positive control in the assay.

The 9 samples were amplified as described in
Example 5(b) and the obtained material was immobilised
to Dynabeads M280-streptavidin.

30 The recombinant fusion protein consisting of the
lac repressor enzyme conjugate (Example 5(c)) was added.

The beads, with the immobilized DNA, were mixed
together with 200 μ l of desalted fusion protein (LacI-
lacZ, OD=0.2) and 200 μ g of sonicated herring sperm DNA
35 in a Eppendorf tube for 30 minutes. The beads was
washed with the TST-buffer. The substrate, ortho-
nitrophenyl- β -D-galactoside (ONPG), was added and the

change of absorbance at room temperature was analyzed. One unit is defined as the change of one A unit per minute at 405 nm.

5 The results shown in Figure 21 demonstrates a good correlation between the DIANA assay and the conventional cell culture assay. The absorbences for the negative samples are very low and confirms the low background levels obtained after a two step PCR.

(e) Solid phase DNA sequencing of positive samples

10 The positive samples obtained by the DIANA assay (Fig. 21) were all directly sequenced using the solid phase procedure outlined in Figure 18. One strand of the bound DNA fragment and the enzyme conjugate were eluted simultaneously with alkali and the remaining
15 immobilized strand was used as template for genomic sequencing. A fluorescent primer corresponding to the lac operator sequence was used.

The DNA was sequenced by melting the strands with 0.15 M NaOH at room temperature in 10 minutes. The
20 beads were thereafter washed three times with 50 μ l TE buffer (0.1 M Tris-HCl, pH=7.5, 1 mM EDTA). For the annealing of primer a buffer containing: 10 mM Tris-HCl, pH=7.5, 100 mM NaCl, 10 mM MgCl₂ and 0.1 mg/ml BSA was mixed together with 2 pmole RIT43 primer in a total
25 volume of 17 μ l. The annealing mixture was heated at 65°C and allowed to cool to room temperature. To the mixture was thereafter 1 μ l of MID solution (Pharmacia, Sweden) and 3 units of T7 DNA polymerase (Pharmacia, Sweden) added to total volume of 20 μ l. To 2.5 μ l of
30 Pharmacia's nucleotide mixtures (A, C, G and T) was 4.5 μ l of the solution above mixed and incubated in 10 minutes at 37°C. The beads were washed with H₂O and incubated at 85°C with 3 μ l of deionized formamide to denaturate the strands. 4 μ l of the generated single
35 strand were loaded on a 7% polyacrylamide gel on the A.L.F. sequencing apparatus (Pharmacia LKB AB, Sweden).

A clear sequence was obtained for all positive

samples and the results for one of the samples (number 1 in Figure 21) is shown in Figure 22.

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CLAIMS:

1. A method of diagnosis of medical conditions by identification of specific DNA characterized in that DNA
5 in a sample is subjected to initial amplification by the polymerase chain reaction (PCR) method using a first pair of primers specific to the target DNA and the amplified DNA, so produced is further amplified by the PCR method using a second primer pair, one or both of
10 which are different from either of said first primer pair and are specific to a sequence or sequences of the target DNA between the hybridisation sites of said first primer pair, one of the primers of said second primer pair being immobilised on a solid support or being
15 provided with means for subsequent attachment to a solid support and the other of said second pair of primers carrying a label or being provided with means for subsequent attachment to a label, said amplification being followed by separation of said solid support
20 carrying said amplified DNA and detection of label attached thereto, one or both of said means for subsequent attachment comprising a distal DNA sequence carried by the said primer which does not hybridise with the target DNA but has a selective affinity for a
25 binding partner attached to either a solid support or a label.
2. A method as claimed in claim 1 in which the solid
30 support comprises magnetic particles.
3. A method as claimed in claim 2 in which the solid support comprises monodisperse, superparamagnetic particles.
- 35 4. A method as claimed in claim 1 in which the solid support is a microtitre well, a dipstick, non-magnetic particles, fibres or capillaries.

5. A method as claimed in any of the previous claims in which the primer or the amplified DNA is attached to the solid support by biotin/avidin or
5 biotin/streptavidin binding.
6. A method as claimed in any of the preceding claims in which the primer or amplified DNA is attached to the label by binding between said DNA and a DNA binding
10 protein carrying said label.
7. A method as claimed in claim 6 in which the DNA binding protein is LacI and the DNA to which it binds is the Lac operon.
15
8. A method as claimed in claim 7 in which the LacI protein is fused to an enzyme label.
9. A method as claimed in any of the preceding claims in which the binding partner for said distal DNA
20 sequence is a third stage DNA primer complementary with said distal DNA sequence and is added to the PCR reaction followed by one or more PCR cycles whereby said third stage primer with attached support or label is
25 incorporated into the amplified target DNA.
10. A method as claimed in any of the preceding claims in which the double stranded amplified target DNA immobilised on said solid support is converted to single
30 stranded form and is subjected to sequencing.
11. A method as claimed in claim 10 in which said distal DNA sequence is the Lac operon so that the amplified target DNA incorporates said Lac operon at the
35 3'-end, detection of the amplified DNA is effected by addition of LacI-fused to an enzyme label and, after removal of contaminants, the enzyme is caused to

generate a signal; after said detection, the double stranded immobilised DNA is subjected to strand splitting and the immobilised single stranded DNA so formed is then subjected to sequencing.

5

12. A kit for performing the method of claim 1 comprising at least the following components:

- 10 (a) a solid support such as a microtitre well or an array of such wells, a dipstick or beads, more preferably magnetic beads, the support carrying (i) means for attachment to amplified target DNA (ii) means for attachment to a primer or (iii) a primer;
- 15 (b) a label carrying (i) means for attachment to amplified target DNA (ii) means for attachment to a primer or (iii) a primer;
- 20 (c) a pair of outer primers and at least one inner primer provided with a DNA handle, where these are not attached to the support or label as specified in (a) or (b) above;
- 25 (d) a polymerase which is preferably heat stable, for example Taq polymerase;
- (e) buffers for the PCR reaction; and
- 30 (f) wash buffers for washing the support carrying immobilised DNA.

Staphylococcal protein A gene

FIG.1

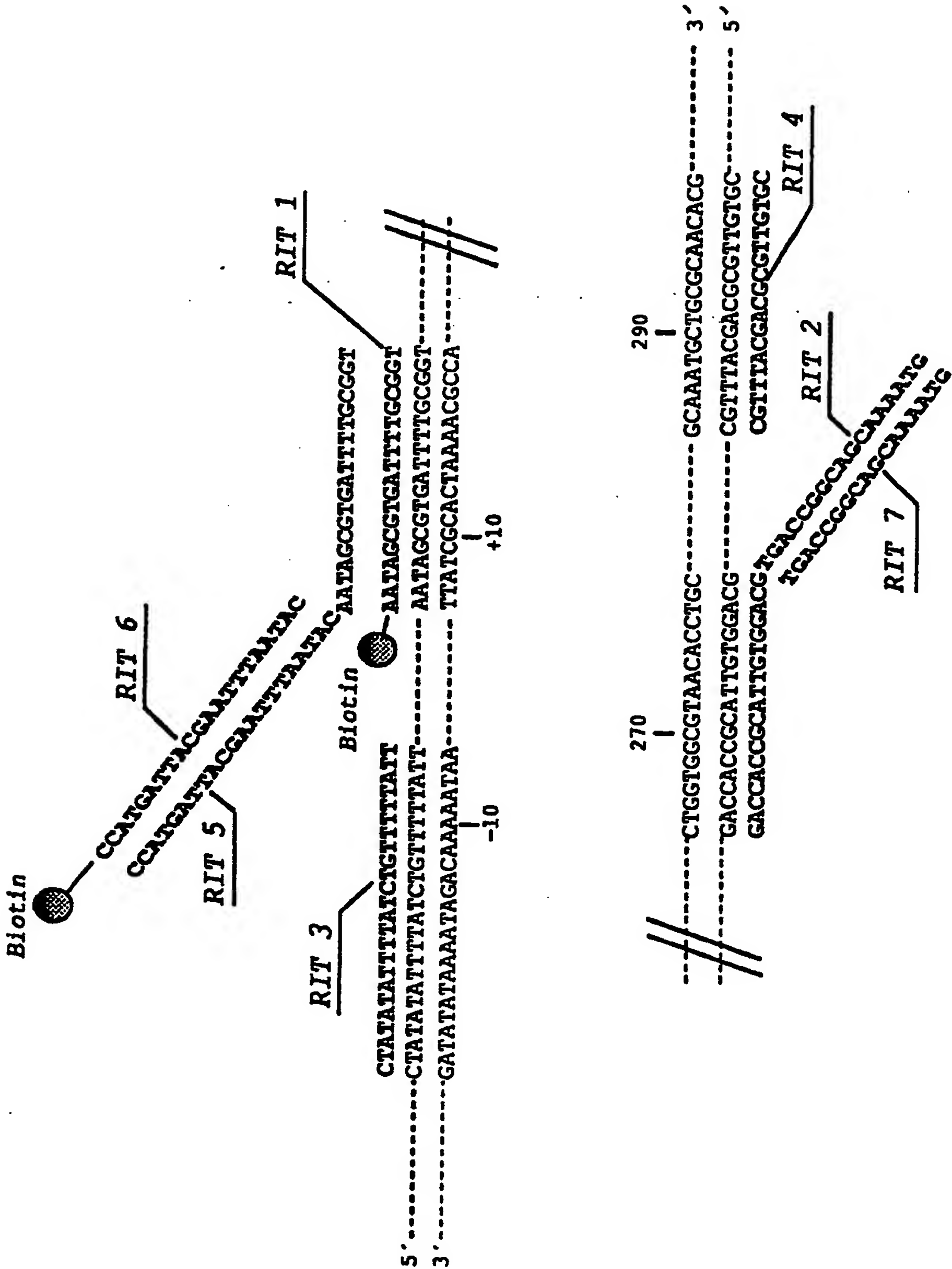
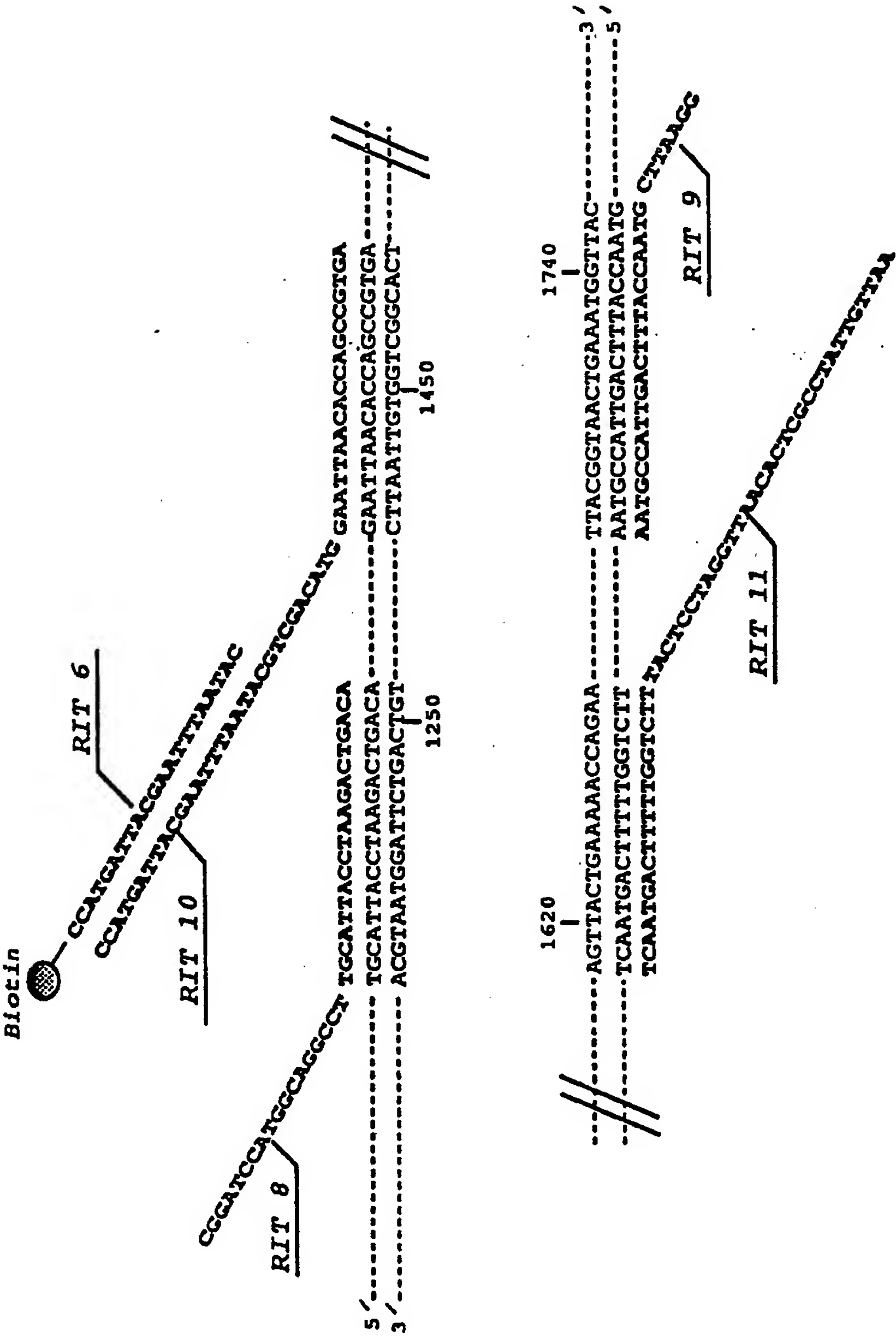


FIG. 2

Streptococcal protein G gene



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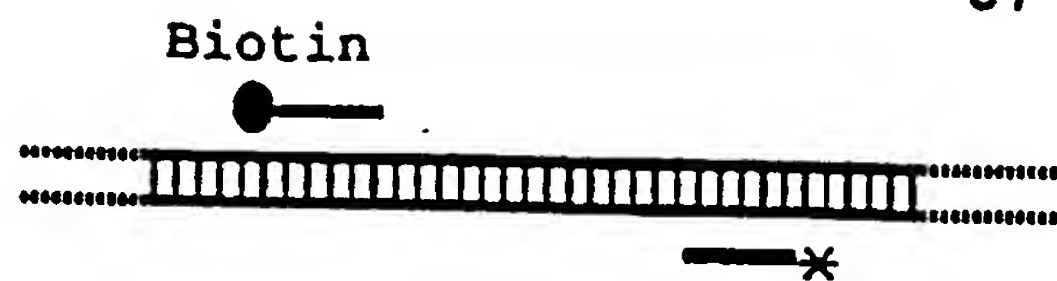


FIG. 3

PCR



Bind to solid support

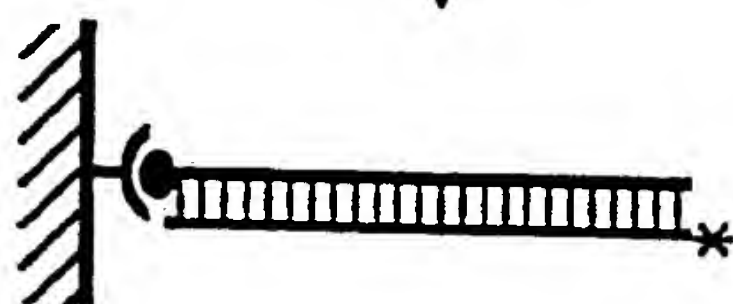
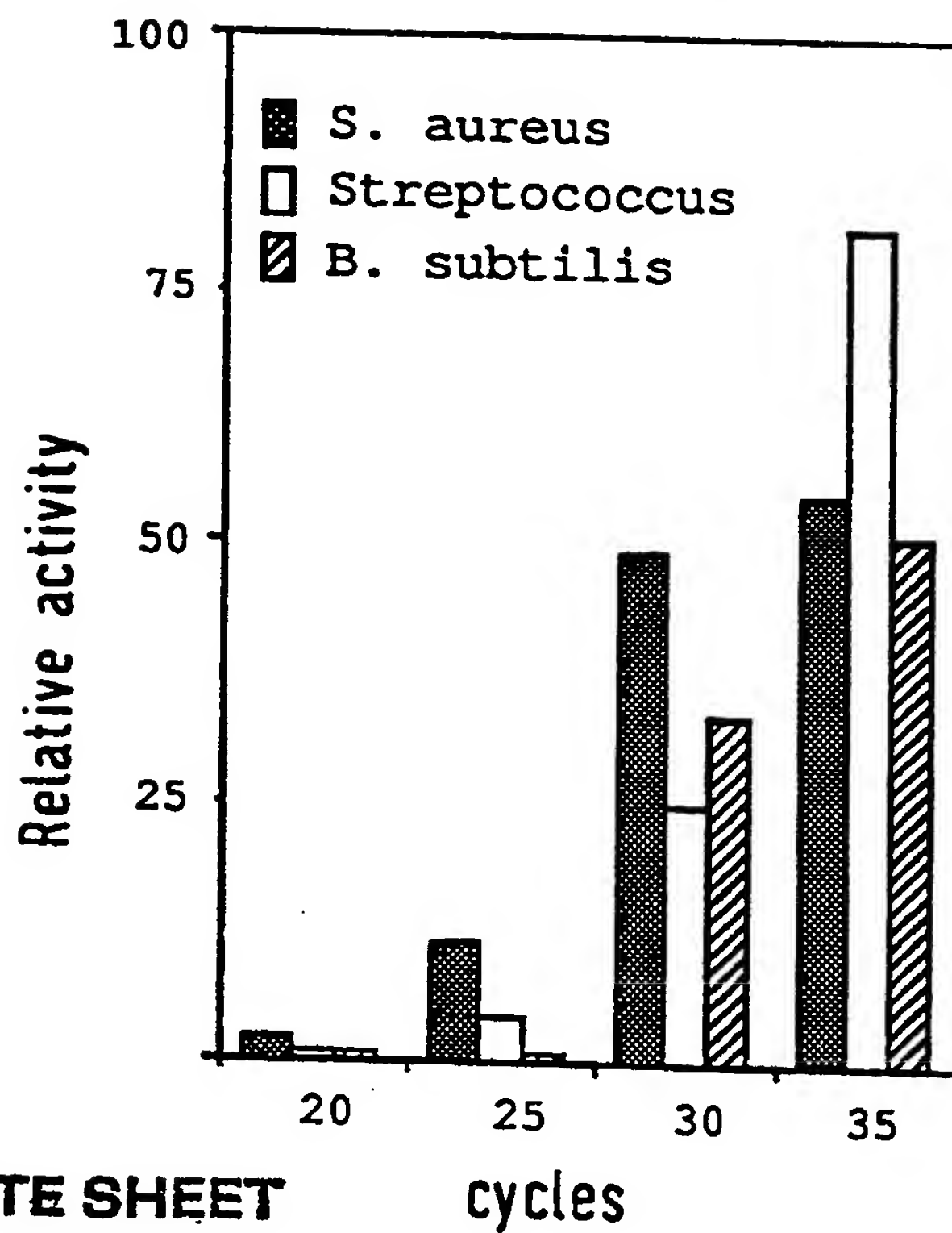


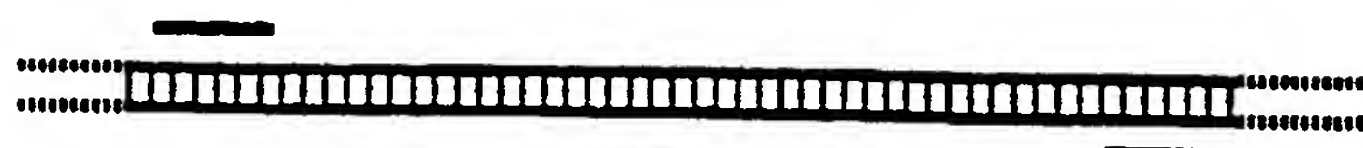
FIG. 4



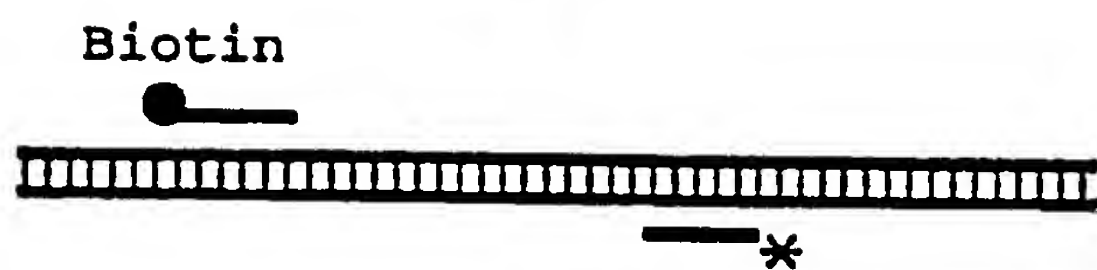
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cycles

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PCR



PCR

Bind to solid support

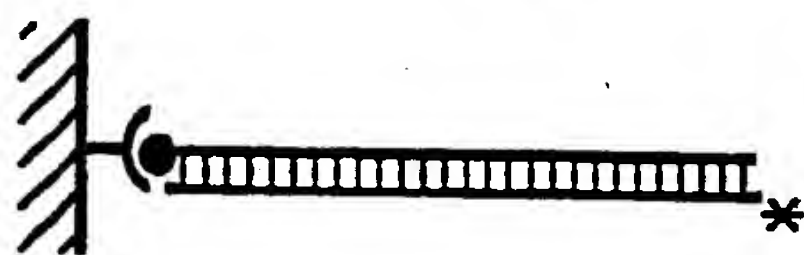
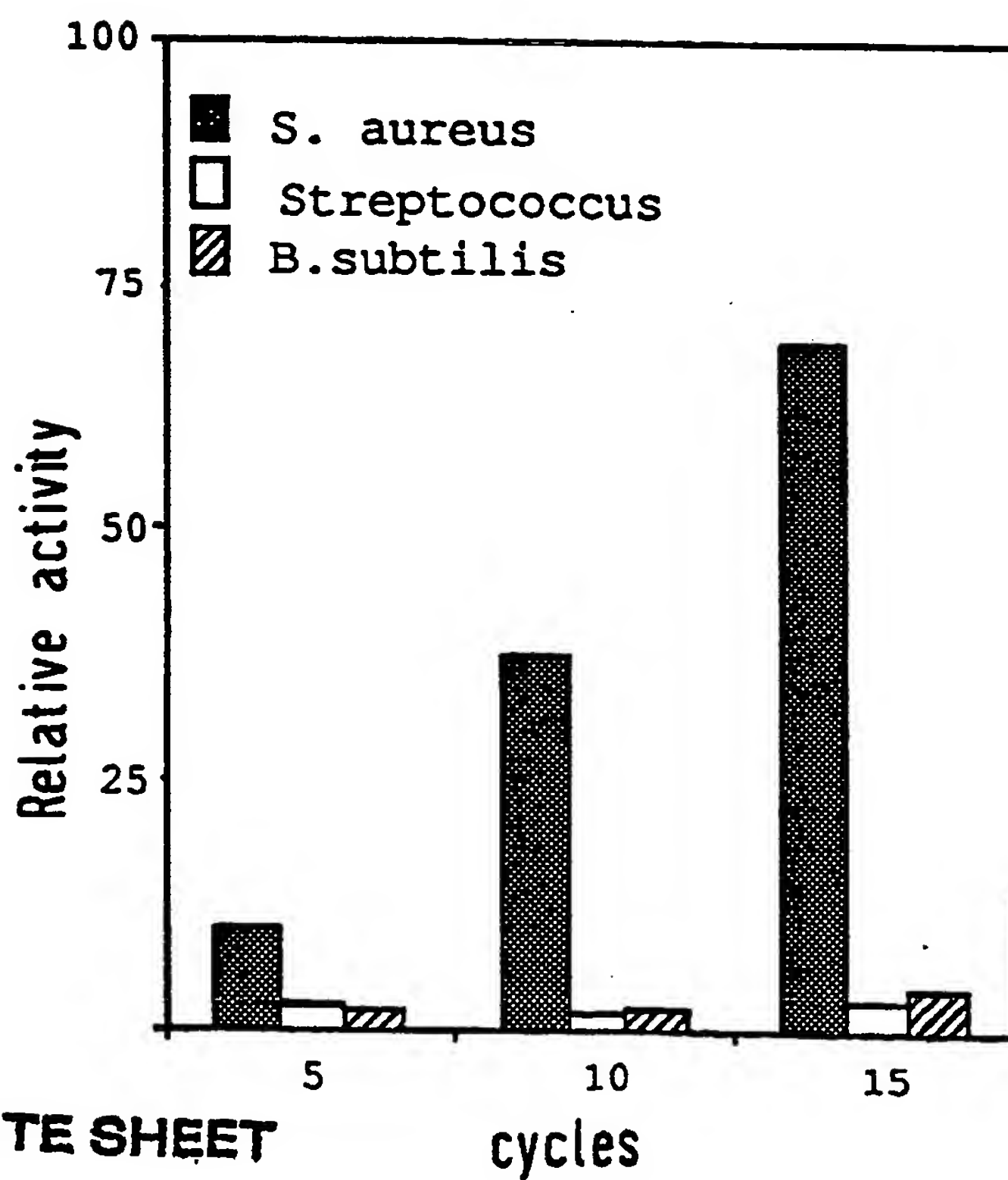


FIG.5

FIG.6



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FIG.7

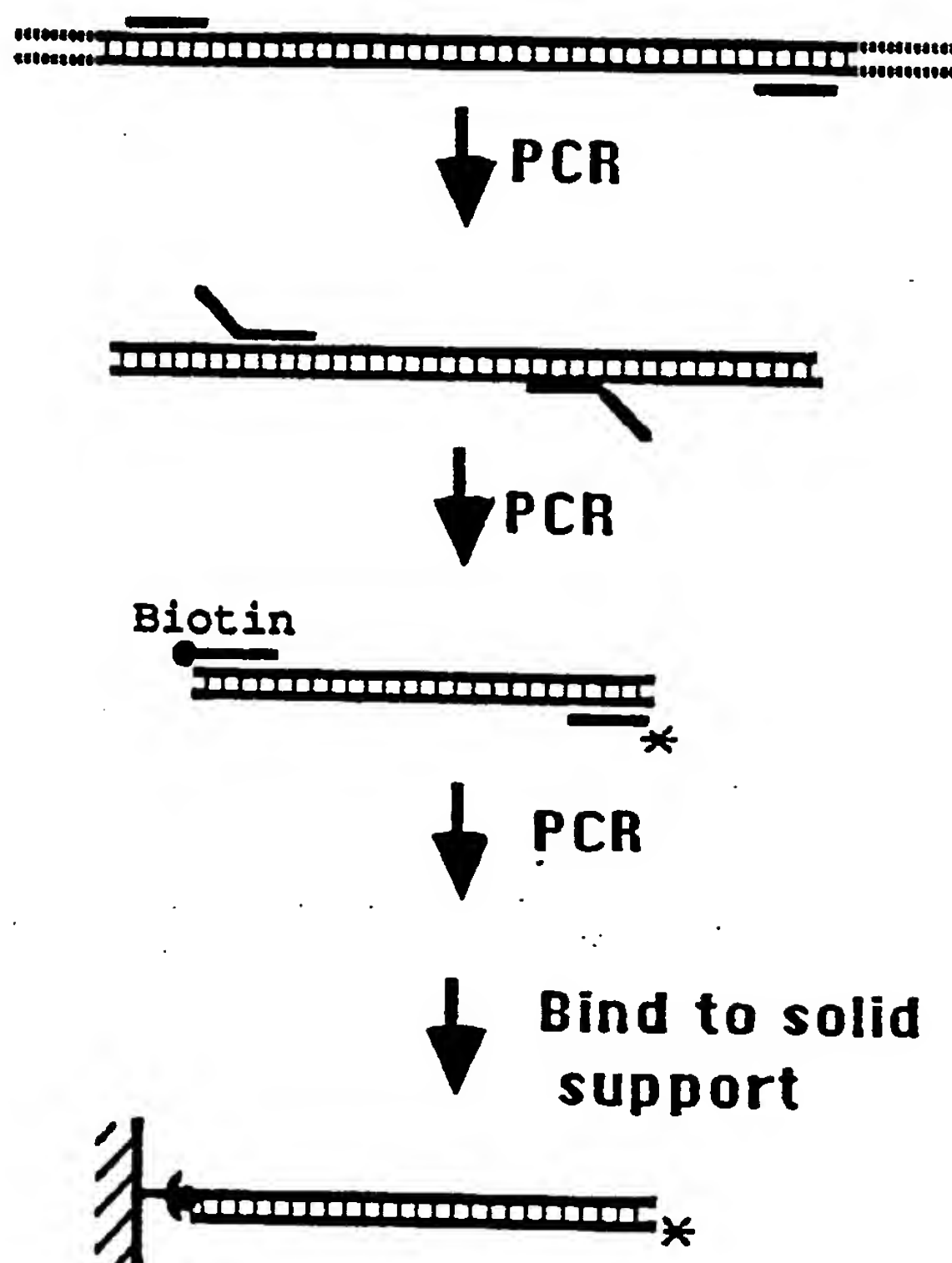
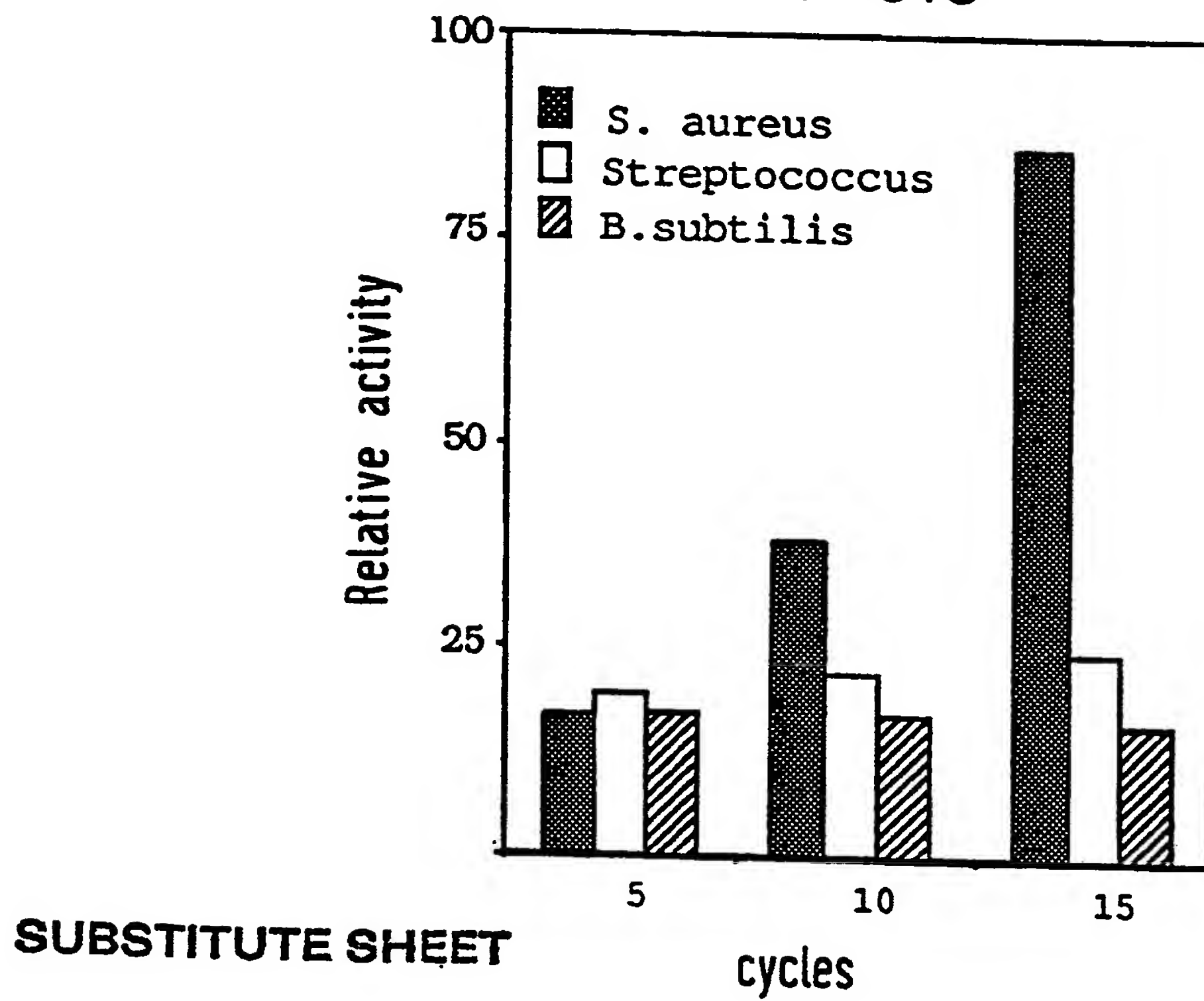
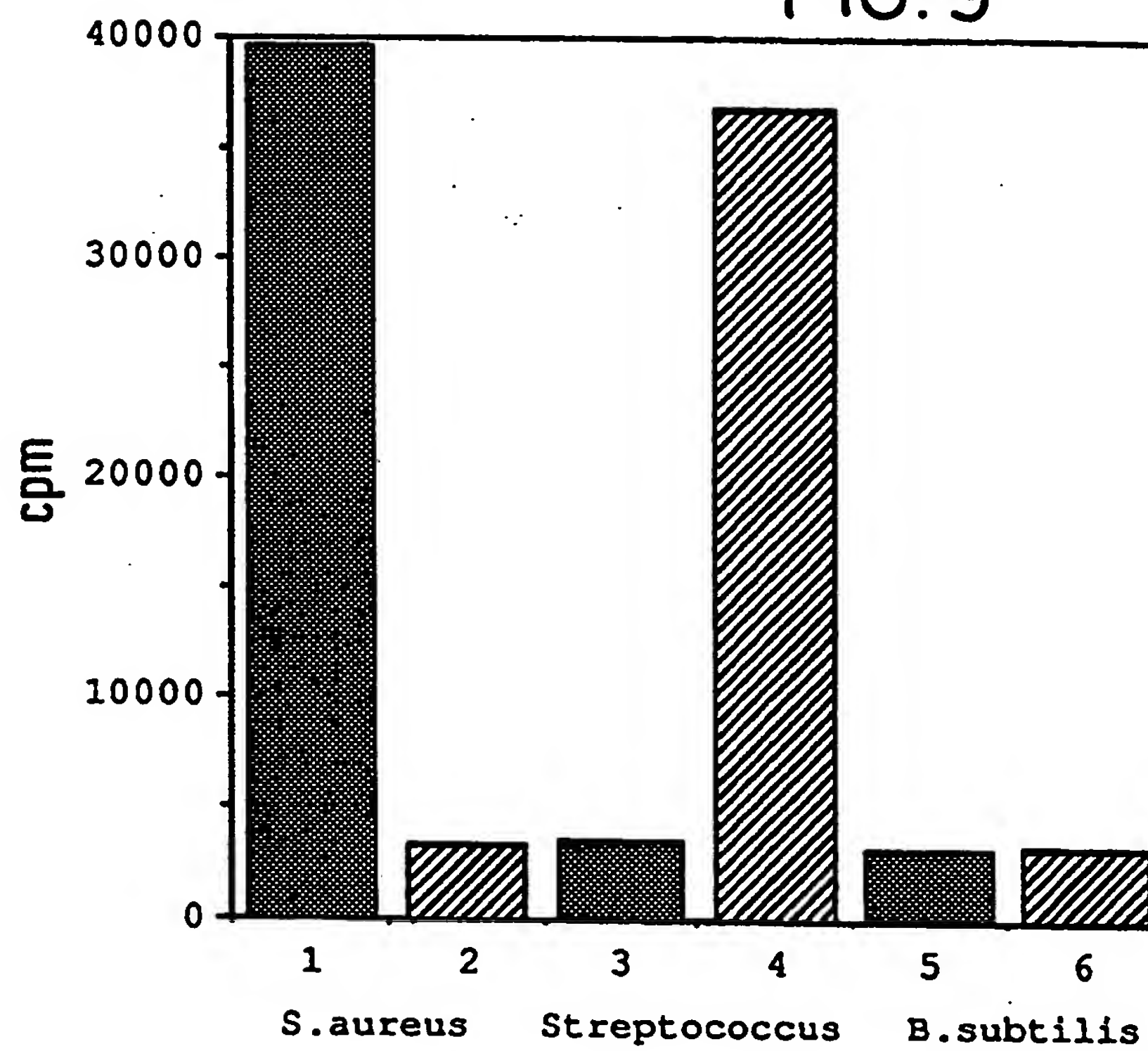


FIG.8



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FIG. 9



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B. Detection of PCR amplified DNA fragments

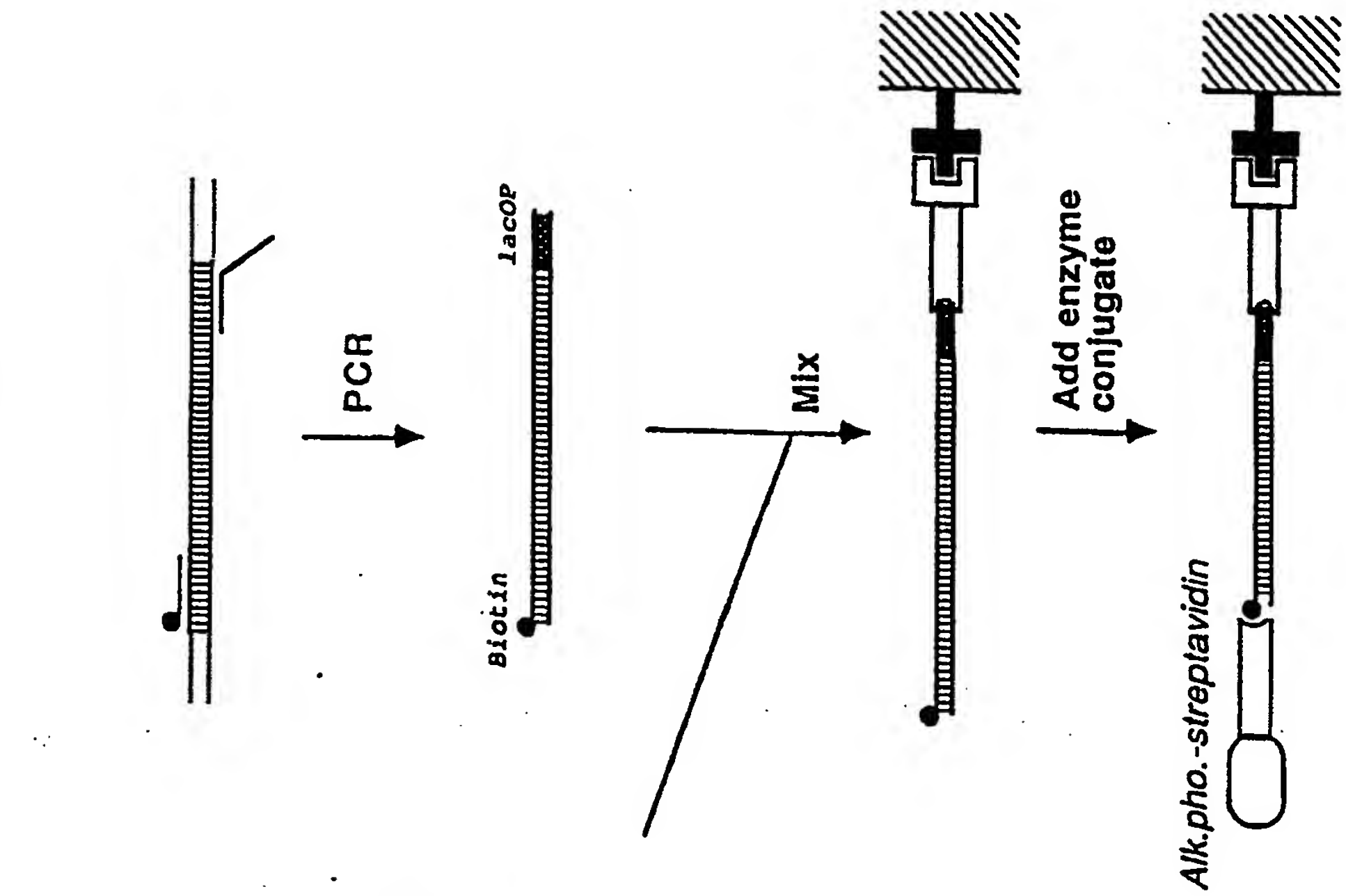
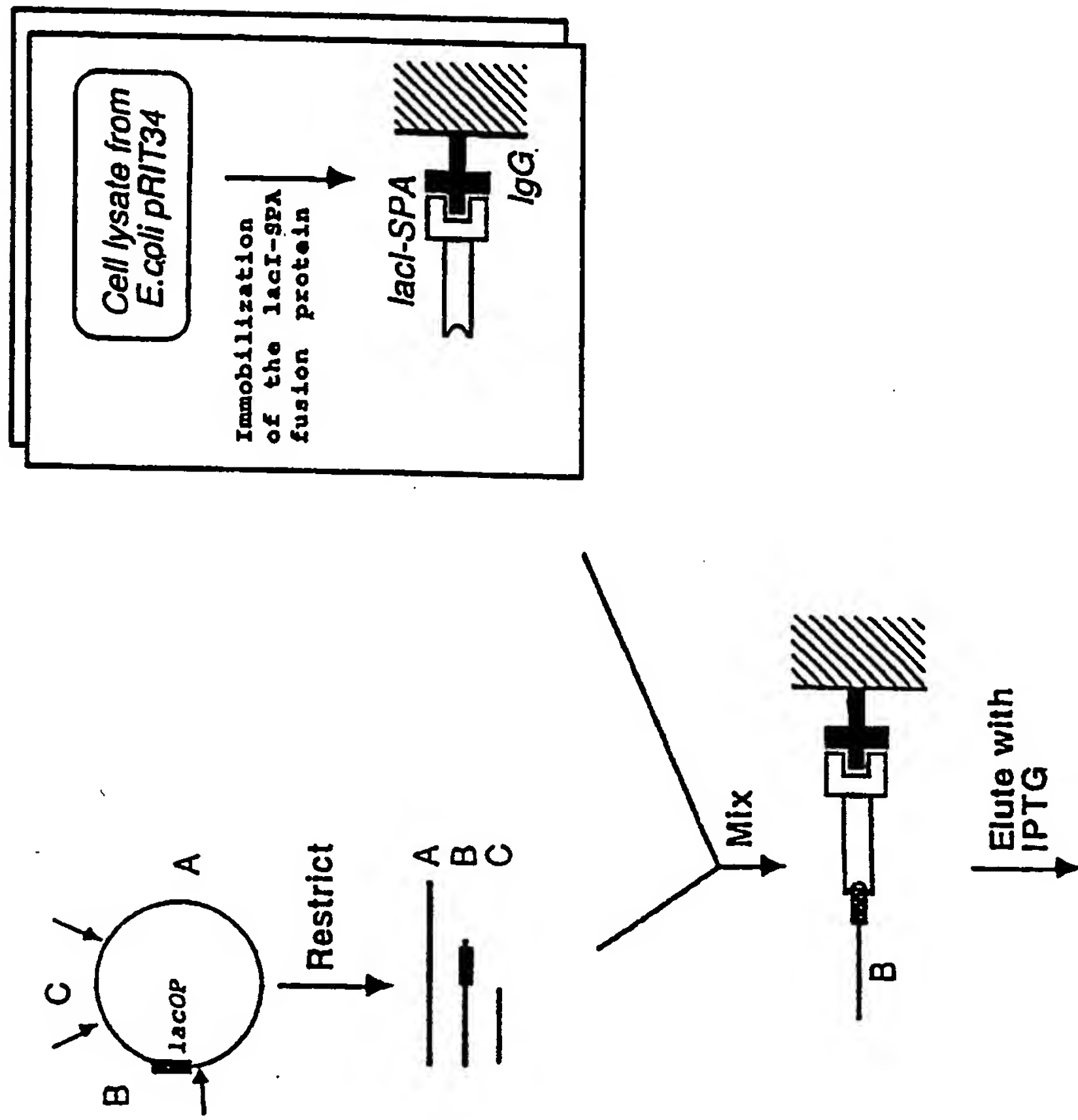


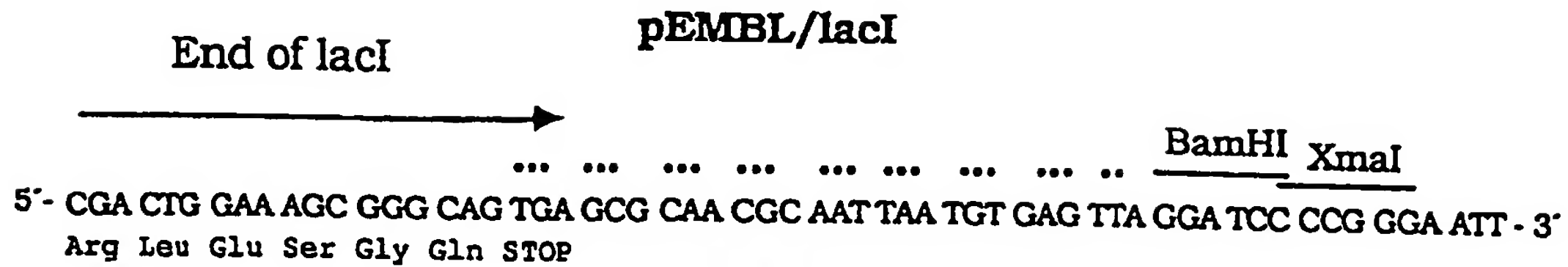
FIG.10

A. Purification of DNA fragments



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FIG. 11



↓
In vitro mutagenesis
by synthetic oligonucleotide

pEMBL/lacI Δ STOP

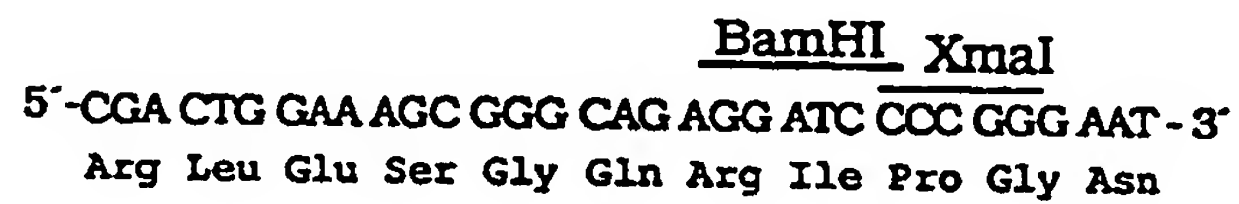
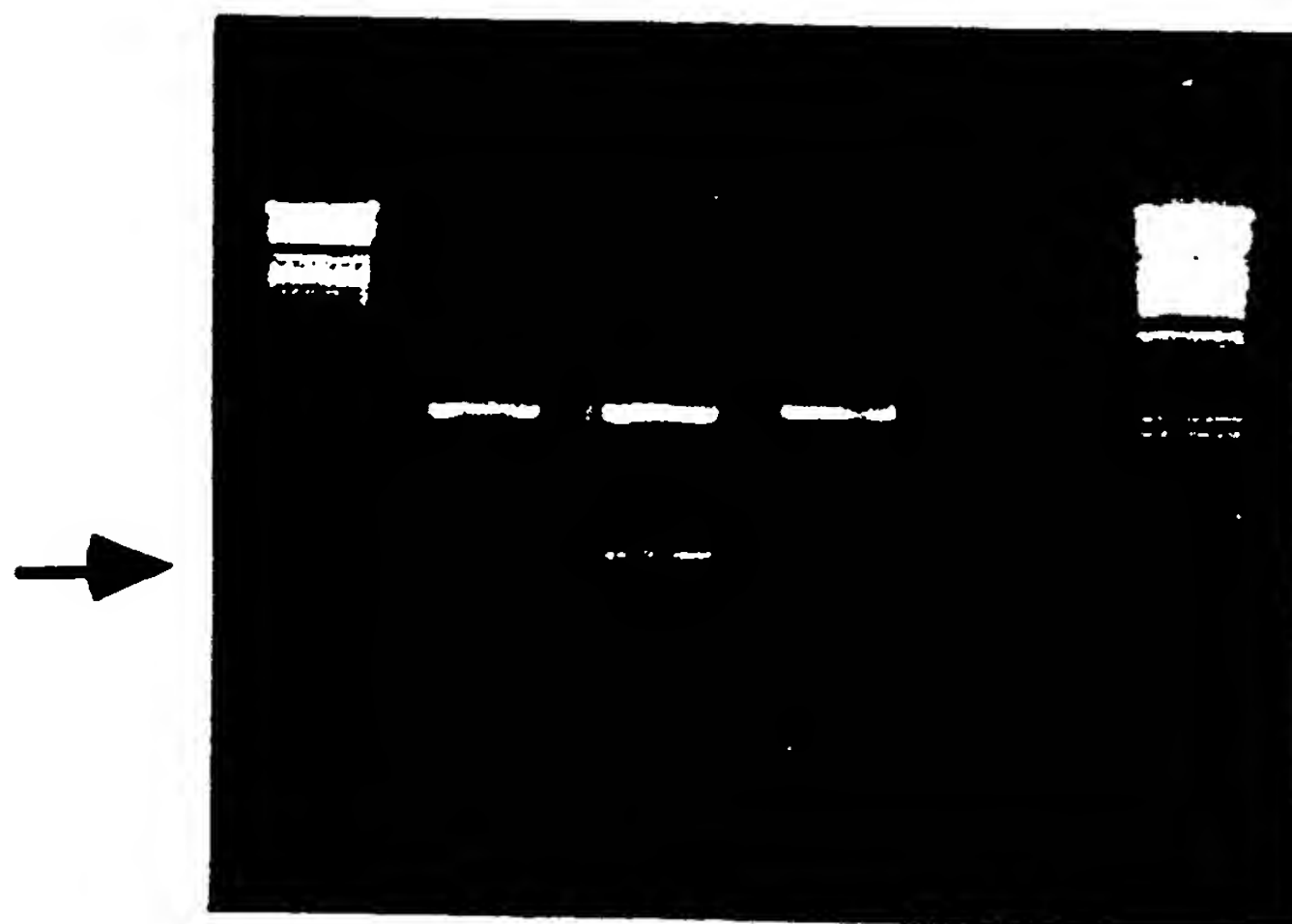
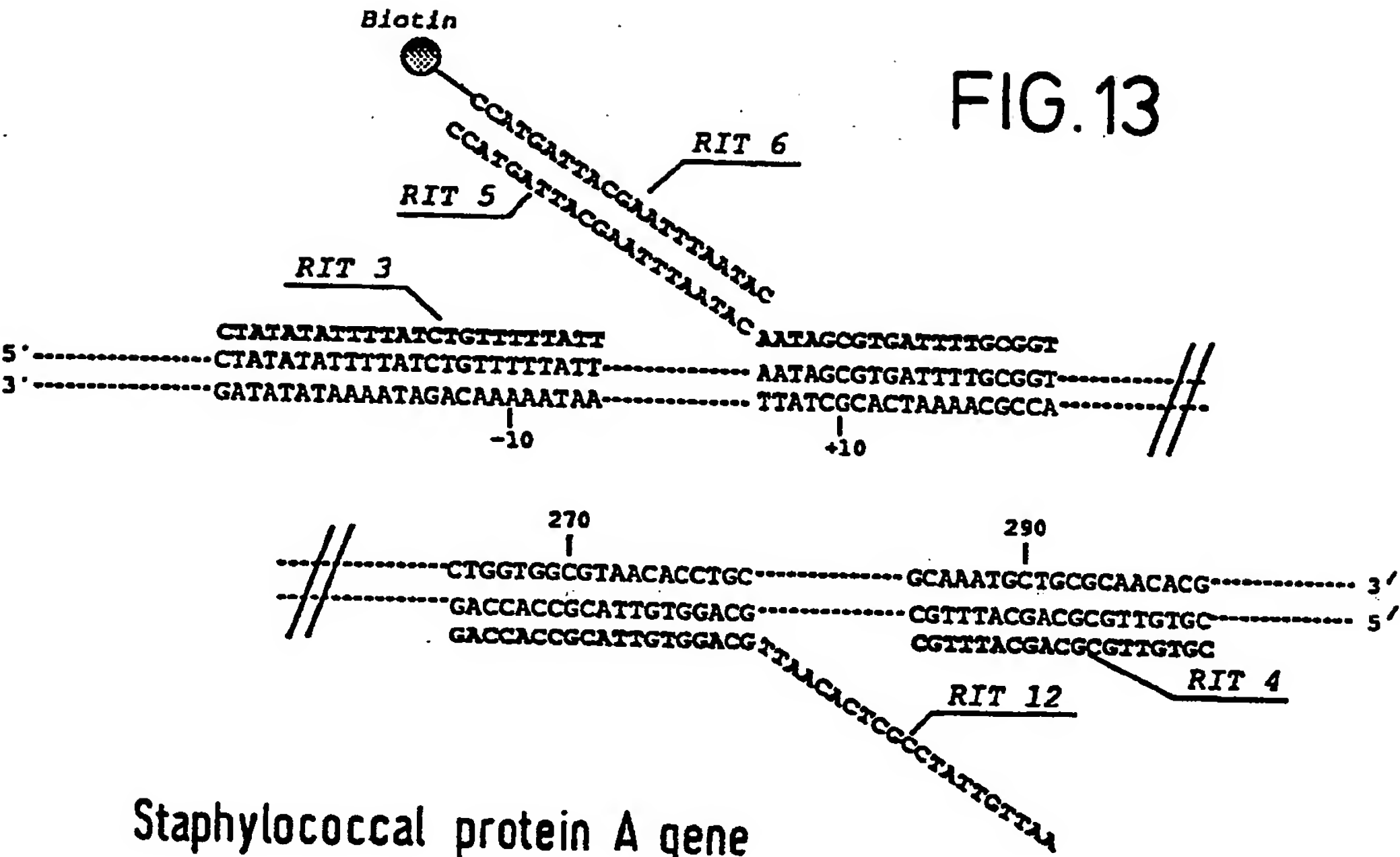


FIG. 12

M 1 2 3 4 M



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Staphylococcal protein A gene

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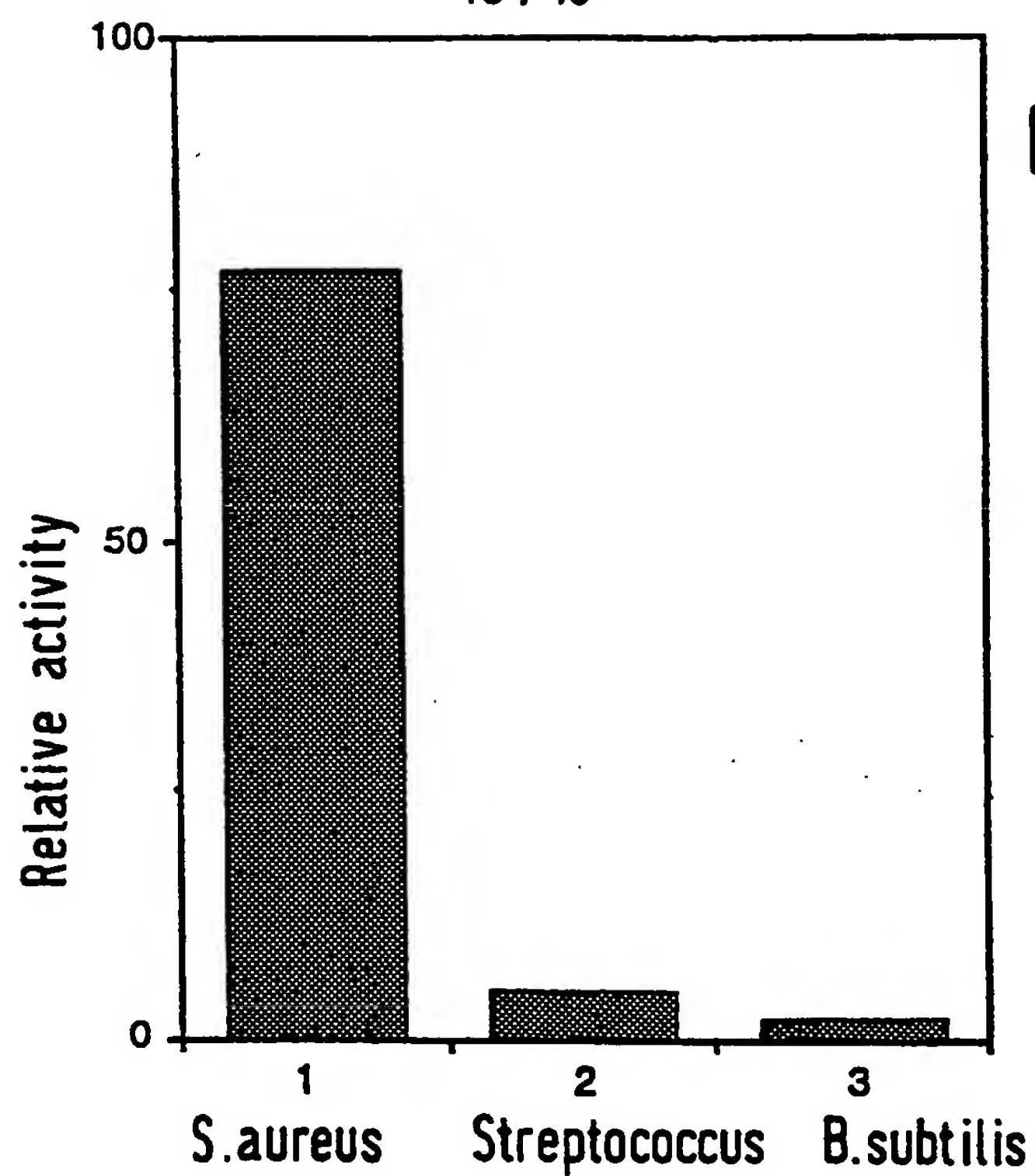


FIG.14

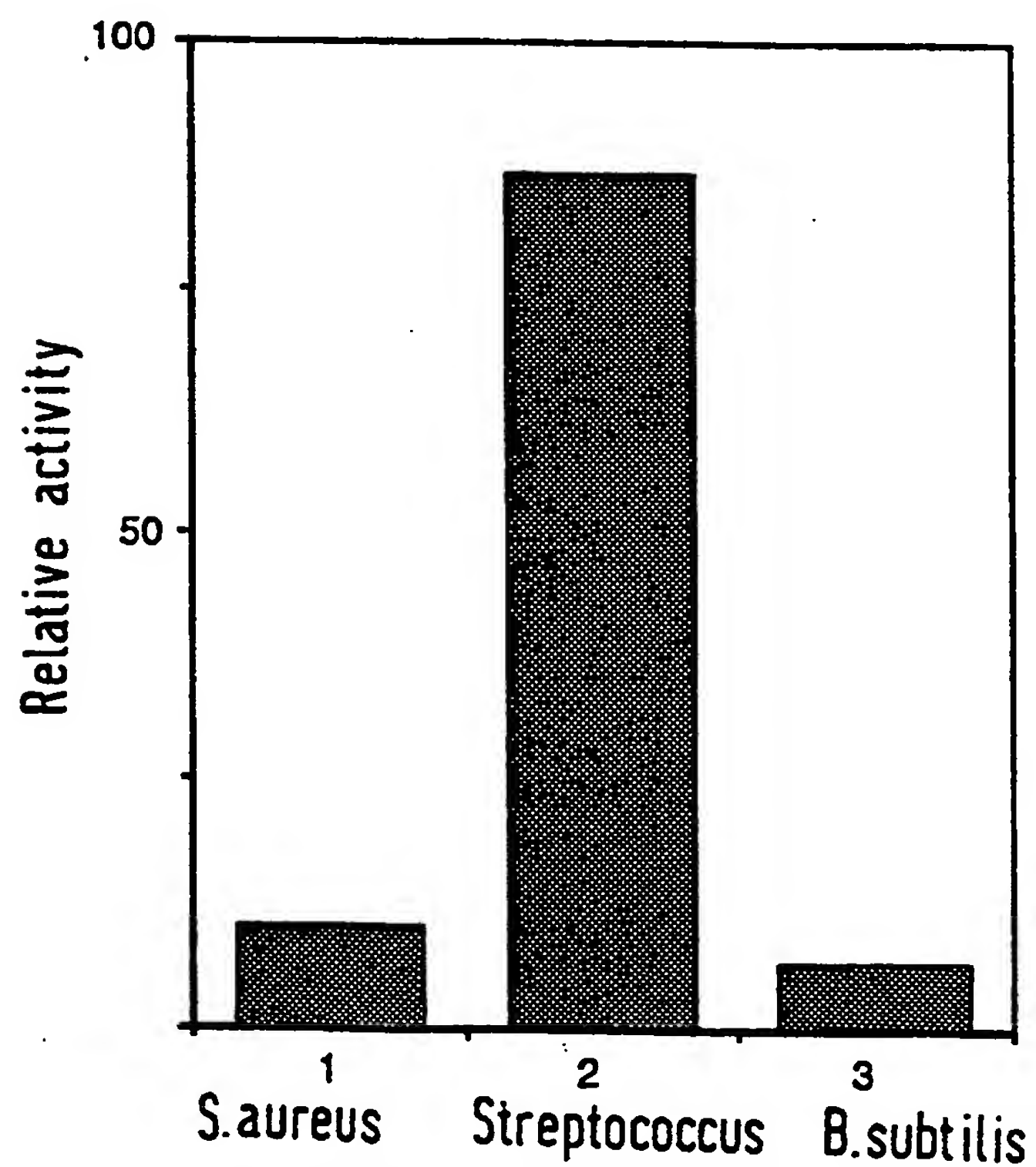


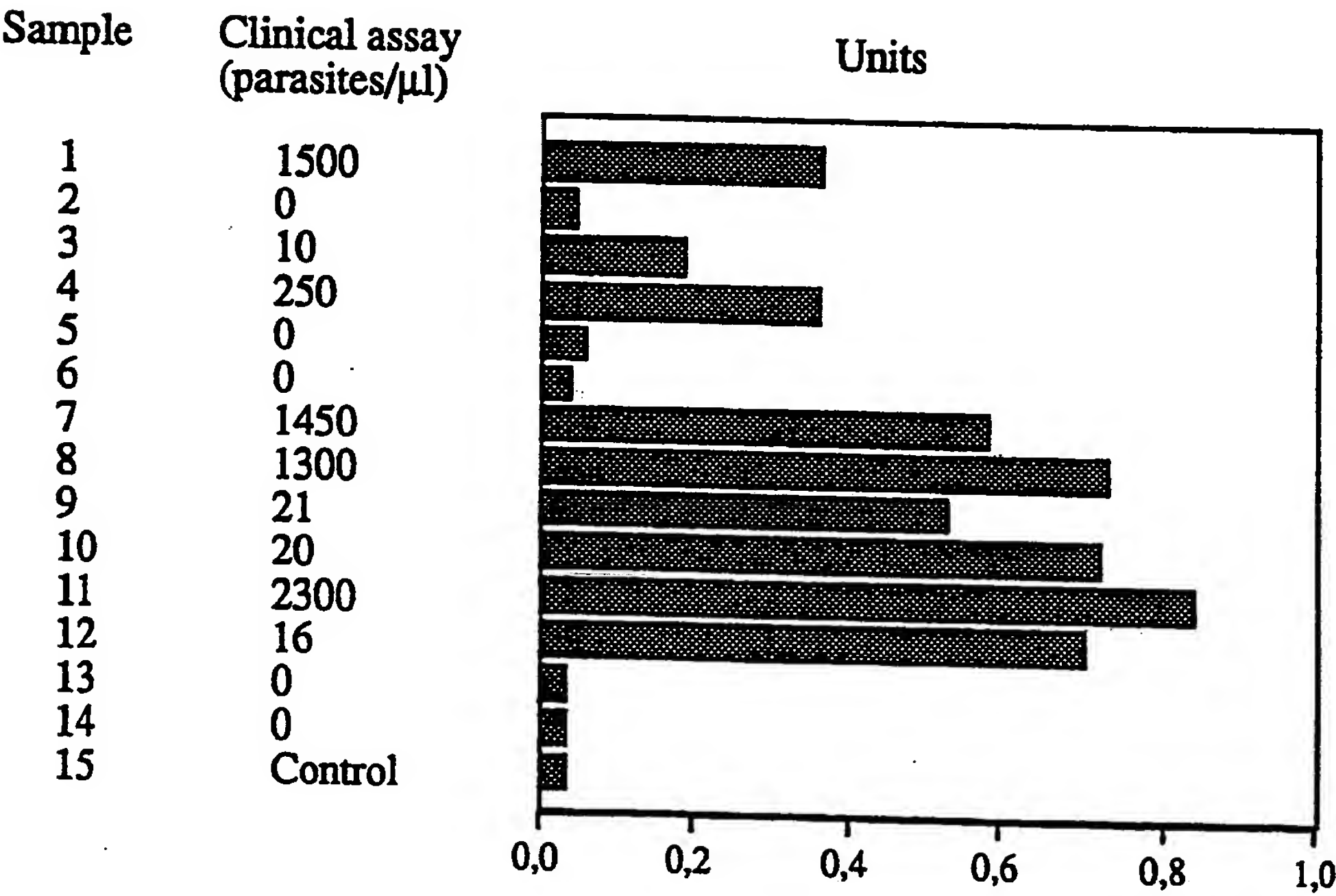
FIG.15

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FIG. 16

RIT33	<i>P. falciparum</i>	+1377	Flanking primer	5'-CCTCCTGATATTGATCATAC-3'
RIT34		+1804		5'-AATATTCTGCCTGTACCAG-3'
RIT35	:	+1421	Inner primer	5'-CCATGATTACGAATTTAATACGAATTCGTTCATGACTGATGTAAATA-3'
RIT36	-	+1761		5'-AATGGTATCCGCTCACAATTAAGCTTCTTTCTTCAAGGTTCTCTCC-3'
RIT6			General biotinylated primer	5'-biotin-CCATGATTACGAATTTAATAC-3'
RIT43	<i>lucOP</i>		General fluorescent primer	5'-FITC-AATGGTATCCGCTCACAATT-3'

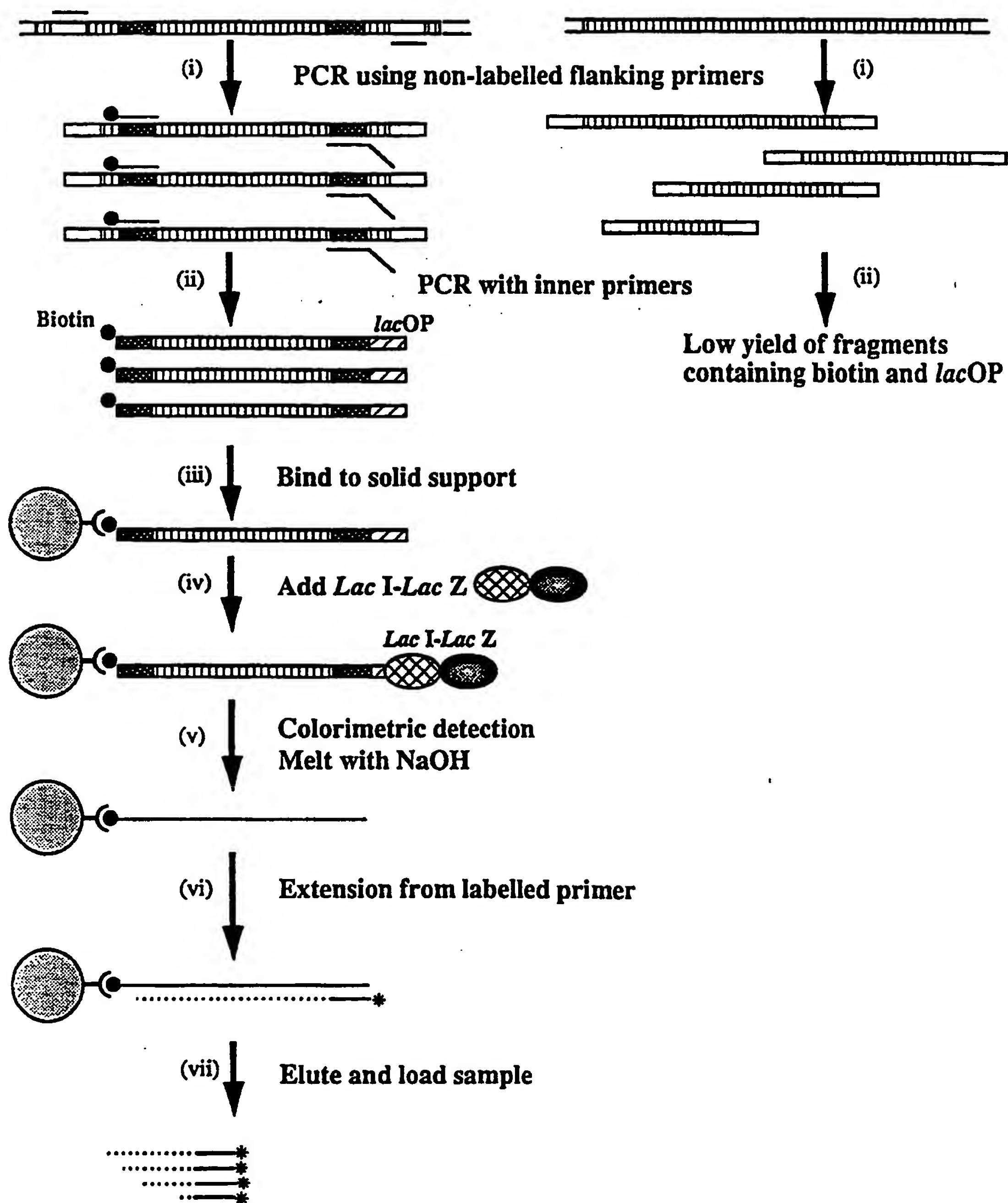
FIG. 17



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FIG.18

Specific target DNA

Non-specific DNA



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FIG.19

RIT 23																RIT 25															
Biotin																GGA CAT AAA AAT ACA GCA AGG															
1013	GCA	ATG	GTT	TCT	TAC	TGT	GGA	Gly	His	Lys	Asn	Thr	Ala	Ser	GTA	ACA															
265	Ala	Met	Val	Ser	Tyr	Cys	Gly	Gly	His	Lys	Asn	Thr	Ala	Ser	Val	Thr															
T																															
1062	ACT	GTG	ATC	AAC	GAG	CCT	TGC	GTA	CAA	GTA	AGT	ATT	GCA	GGA	GCA	GAT															
281	Thr	Val	Ile	Asn	Glu	Pro	Cys	Val	Gln	Val	Ser	Ile	Ala	Gly	Ala	Asp															
1108	TGG	TCT	TAT	GTT	TGT	AAG	CCT	GTA	GAA	TAT	GTG	ATC	TCC	GTT	TCC	AAT															
297	Trp	Ser	Tyr	Val	Cys	Lys	Pro	Val	Glu	Tyr	Val	Ile	Ser	Val	Ser	Asn															
1157	CGT	GGA	GAT	CTT	GTG	TTG	CGA	GAT	GTC	GTC	GTT	AAA	GAC	ACT	CTT	TCT															
313	Pro	Gly	Asp	Leu	Val	Leu	Arg	Asp	Val	Val	Val	Lys	Asp	Thr	Leu	Ser															
G																															
1205	CCC	GGA	GTC	ACA	GTT	CTT	GAA	GCT	GCA	GGA	GCT	CAA	ATT	TCT	TGT	AAT															
329	Pro	Gly	Val	Thr	Val	Leu	Glu	Ala	Ala	Gly	Ala	Gln	Ile	Ser	Cys	Asn															
RIT 26																Lac op															
1253	AAA	GTA	GTT	TGG	ACT	GTG	AAA	GAA	CTG	AAT	CCT	GGA	GAG	TCT	CTA	CAG															
345	Lys	Val	Val	Trp	Thr	Val	Lys	Glu	Leu	Asn	Pro	Gly	Glu	Ser	Leu	Gln															
RIT 24																CTA GTA AGA GCA CAA ACT CCT															
1301	TAT	AAA	GTT	CTA	GTA	AGA	GCA	CAA	ACT	CCT	GGA	CAA	TTC																		
361	Tyr	Lys	Val	Leu	Val	Arg	Ala	Gln	Thr	Pro	Gly	Gln	Phe																		

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FIG. 20

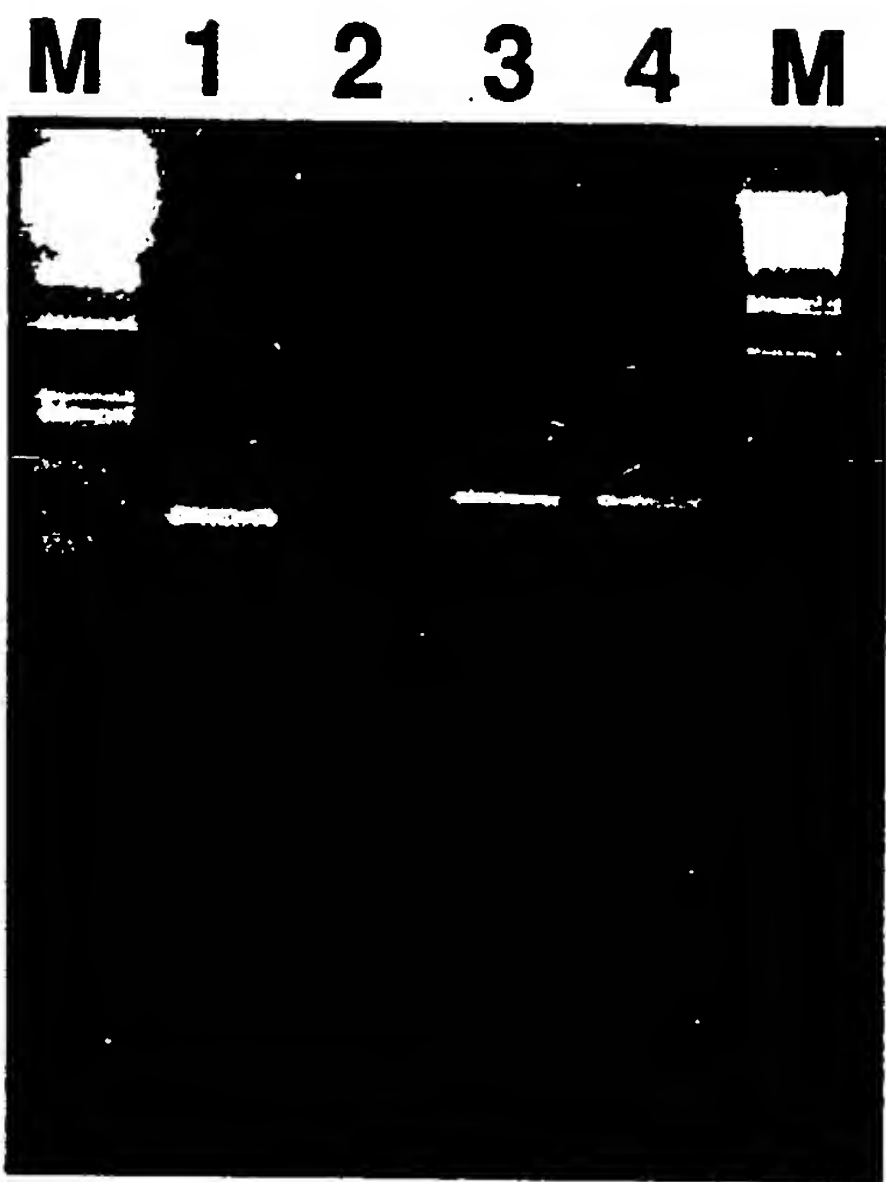
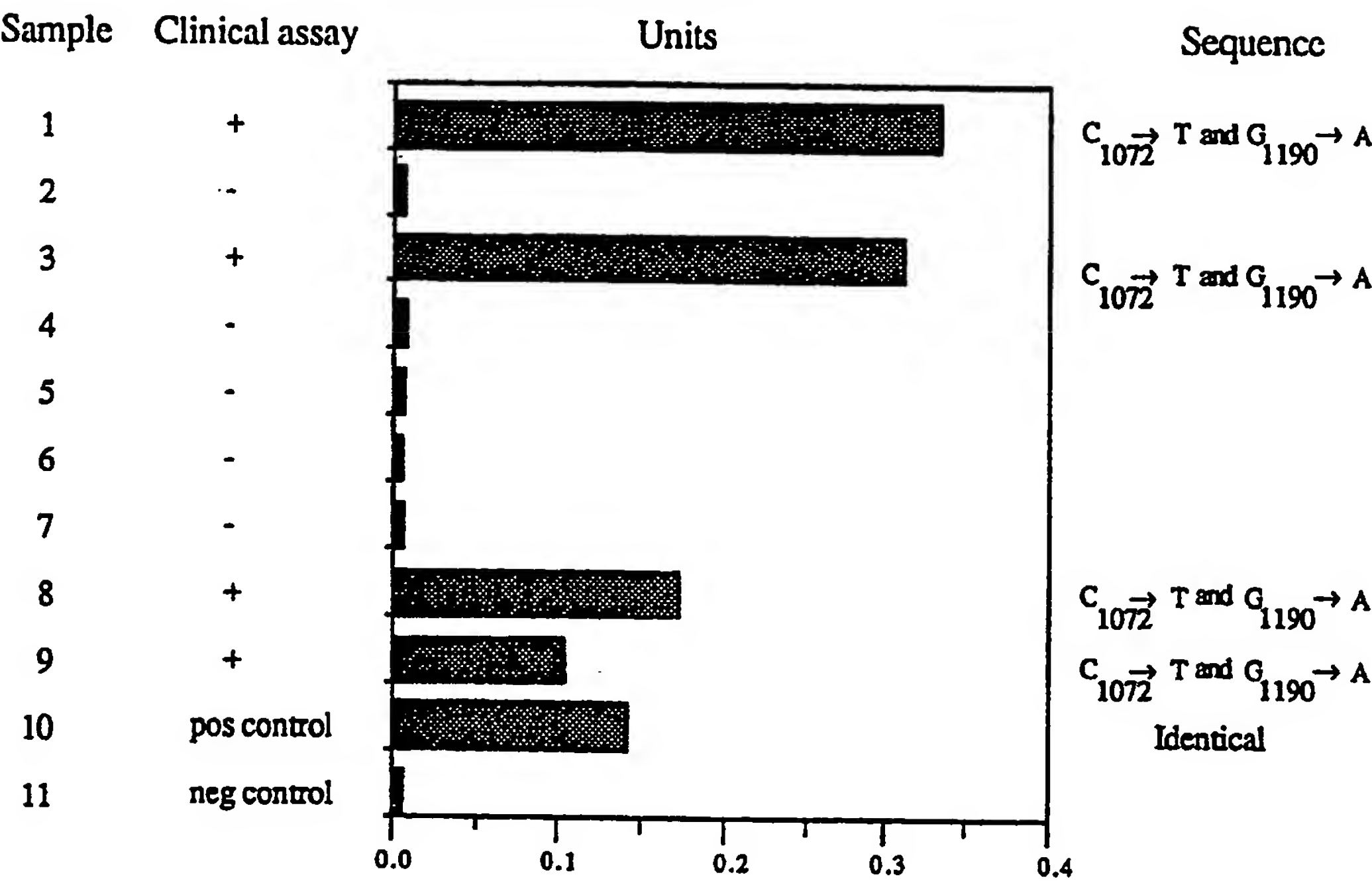
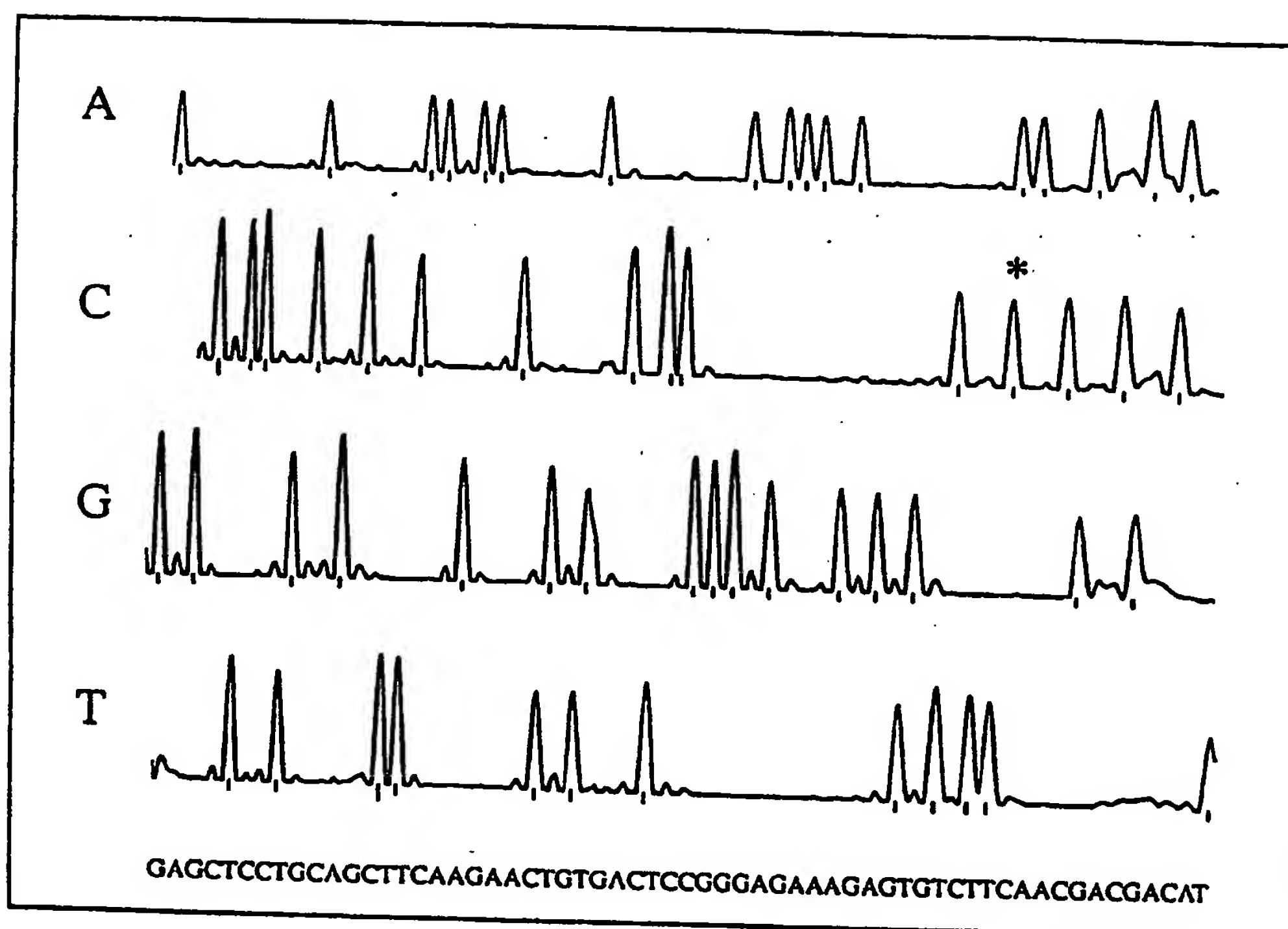


FIG. 21



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FIG. 22



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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 90/00454

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : C 12 Q 1/68																	
II. FIELDS SEARCHED <div style="text-align: right; font-size: small;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;">Classification System :</td> <td style="width: 50%; border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; padding-top: 10px;">IPC⁵</td> <td style="border: none; padding-top: 10px;">C 12 Q</td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 10px;"> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ </div>			Classification System :	Classification Symbols	IPC ⁵	C 12 Q											
Classification System :	Classification Symbols																
IPC ⁵	C 12 Q																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse; font-size: x-small;"> <thead> <tr> <th style="width: 10%;">Category ⁹</th> <th style="width: 70%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="vertical-align: top;"> Nucleic Acid Research, volume 16, no. 23, December 1988, IRL Press Ltd, (Oxford, GB), A.-C. Syvänen et al.: "Quantification of polymerase chain reaction products by affinity-based hybrid collection", pages 11327-11338 see the whole article -- </td> <td style="text-align: center; vertical-align: top;">1,4,5,9, 10,12</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;"> EP, A, 0192168 (MOLECULAR DIAGNOSTICS INC.) 27 August 1986 see page 5, line 2 - page 8, line 20; figure -- </td> <td style="text-align: center; vertical-align: top;">1-12</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;"> WO, A, 86/05815 (GENETICS INTERNATIONAL INC.) 9 October 1986 see page 6, line 12 - page 8, line 8; page 13, line 10 - page 14, line 19 -- </td> <td style="text-align: center; vertical-align: top;">1-12</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;"> Chemical Abstracts, volume 111, no. 23, 4 December 1989, (Columbus, Ohio, US), ././ </td> <td style="text-align: center; vertical-align: top;">1-12</td> </tr> </tbody> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Nucleic Acid Research, volume 16, no. 23, December 1988, IRL Press Ltd, (Oxford, GB), A.-C. Syvänen et al.: "Quantification of polymerase chain reaction products by affinity-based hybrid collection", pages 11327-11338 see the whole article --	1,4,5,9, 10,12	A	EP, A, 0192168 (MOLECULAR DIAGNOSTICS INC.) 27 August 1986 see page 5, line 2 - page 8, line 20; figure --	1-12	A	WO, A, 86/05815 (GENETICS INTERNATIONAL INC.) 9 October 1986 see page 6, line 12 - page 8, line 8; page 13, line 10 - page 14, line 19 --	1-12	A	Chemical Abstracts, volume 111, no. 23, 4 December 1989, (Columbus, Ohio, US), ././	1-12
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<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 48%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> Date of the Actual Completion of the International Search 12th June 1990 </td> <td style="width: 50%; border: none; vertical-align: top;"> Date of Mailing of this International Search Report <div style="text-align: center; font-size: large; font-weight: bold;">04 JUL 1990</div> </td> </tr> <tr> <td style="width: 50%; border: none; vertical-align: top;"> International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div> </td> <td style="width: 50%; border: none; vertical-align: top;"> Signature of Authorized Officer <div style="text-align: center;"> <div style="text-align: center; font-weight: bold;">MISS T. TAZELAAR</div> </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 12th June 1990	Date of Mailing of this International Search Report <div style="text-align: center; font-size: large; font-weight: bold;">04 JUL 1990</div>	International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;"> <div style="text-align: center; font-weight: bold;">MISS T. TAZELAAR</div> </div>											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
T	<p>see page 170, abstract 210118f, & US, A, 200876 (UNITED STATES DEPT. OF HEALTH AND HUMAN SERVICES) 15 February 1989</p> <p>--</p> <p>Journal of Cellular Biochemistry, no. 13, part E, M. Uhlén et al.: "Approaches to solid-phase DNA technology using PCR", page 310, abstract WH 250 see the whole abstract</p> <p>-----</p>	1-12

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9000454
SA 35222

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 29/06/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0192168	27-08-86	AU-A- 5329486	28-08-86
		JP-A- 61195699	29-08-86
WO-A- 8605815	09-10-86	AU-A- 5667186	23-10-86
		EP-A- 0216844	08-04-87

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